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Differential Domain Architecture Directs Nedd4 Family E3 Ligase Function.

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Abstract

Nedd4-family E3 ubiquitin ligases regulate signaling in intracellular pathways that control cancer, blood pressure, iron metabolism, and inflammation. These E3 ligases are catalytically active, and share a highly conserved, modular architecture. How Nedd4 family members are differentially regulated, despite their high degree of homology, is unclear. A regulatory mechanism that maintains an inactive state, common to Nedd4 family members, is autoinhibition. The majority of Nedd4 family members are autoinhibited by an intramolecular interaction between the N terminal C2 domain and the HECT domain. One subfamily of Nedd4 family members that includes the E3 ligase Itch does not appear to be regulated by a C2 domain interaction. The molecular mechanism regulating activation of these E3 ligases is unclear. Here I present findings that illustrate a novel intramolecular interaction regulating Itch and related Nedd4 family member activation that distinguish these E3s as functionally distinct from other Nedd4 family members. I have determined that Itch is autoinhibited by an intramolecular interaction between two central WW domains and two regions in its HECT domain. While capable of interacting with an E2 in its autoinhibited state, Itch is unable to accept ubiquitin onto its catalytic cysteine until this WW domain – HECT domain mediated autoinhibition is relieved. Ndfip proteins bind the WW domains of Itch to release the HECT, facilitating the E2-E3 transthiolation reaction and Itch activation. This inhibitory mechanism can be generalized to the closely related family member WWP2 and, by similarity, WWP1. Further, I tested whether multiple PY motifs are required for Ndfip1 to activate Itch, and found that single PY motifs were not sufficient to relieve Itch autoinhibition. This indicates that activators of Itch can be functionally distinguished from substrates. These data establish a novel mechanism for control of the function of a subfamily of Nedd4 E3 ligases at the level of E2-E3 transthiolation, and suggest that Nedd4 family members can be functionally subdivided based on their autoinhibitory mechanisms.

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Christopher R. Riling

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ABSTRACT

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Paula M. Oliver

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Chapter 1 - Introduction

Post-translational modifications facilitate dynamic signaling.

Cells sense and respond to their environment in a controlled, specific, and timely manner. This requires that signaling events be controlled dynamically in both space and time. Control of cell signaling occurs continuously and on many levels. Through adjusting the protein production, type, and removal, cells utilize an interconnected, multi-tiered and intricate regulatory network. Modifying transcription and translation of proteins is a slow process that does not allow a rapid response to stimuli. Rather, post-translational modifications (PTMs) of existing proteins in the cell would allow the cell to detect and respond to its environment in a controlled, immediate and localized manner.

There are over 200 types of post-translational modification that have been recorded(1). To simplify this array, we can consider two important facets of PTMs. First, that they can be either reversible or irreversible. Irreversible PTMs are relatively uncomplicated in the sense of signaling dynamics, as they are essentially deterministic. Reversible PTMs, however, allow for a nuanced signaling stage in which the interplay between modified and unmodified substrate, PTM addition and PTM removal allows for several regulatory tiers. Small changes in the concentration or activity of enzymes that add modifications or remove modifications can result in a 'tipped balance', shifting the homeostasis of modified substrate and resulting in downstream signaling events. This first layer of signaling complexity is complemented by the second facet of the PTM system: proteins can either be modified in a binary manner, in which they are either modified or unmodified, or a variadic manner, in which the modifications can be presented in a variety of distinct states. At the amino acid level, phosphorylation can be considered a binary-type modification. The amino acid that is phosphorylated is modified with one phosphogroup, and this phosphogroup is unvaried in that it is always the same molecular structure and charge. Therefore there are two states in which this amino acid can exist – it is either phosphorylated or unphosphorylated. Alternatively, an example of variadic modification is provided by ADP-ribosylation. Substrates can undergo both mono-ADP-ribosylation, in which a protein is modified with a single ADP-ribose moiety, or poly-ADP-

ribosylation (PARsylation) in which polymers of ADP-ribose monomers are built on substrates (2). These poly-ADP-ribose chains are linked by glycosidic bridges and can appear in linear, branched and heterogeneous arrays, offering a broad variety of distinctly recognizable modifications that can be placed on a single amino acid. Ultimately, these facets of PTMs allow for a highly nuanced, sensitive and robust signaling system in which complicated information can be encoded quickly and appropriately.

The Ubiquitin Cascade

Increasingly complex cells require increasingly complex signaling systems. One method the eukaryotic cell has for meeting this demand is the ubiquitin cascade. The ubiquitin cascade is a stepwise series of enzymatic reactions that takes up free ubiquitin protein from the cytosol and ultimately facilitates the formation of a covalent isopeptide bond between ubiquitin and a target. This ubiquitylation can signal for a variety of substrate fates, including proteasomal degradation, lysosomal degradation, membrane localization, protein complex formation and receptor internalization. Ubiquitin can be covalently attached to multiple residues of other molecules of ubiquitin itself, resulting in the synthesis of polyubiquitin chains with distinct topologies, which will be discussed in further detail below. The ubiquitin cascade exhibits both variadic capacity and reversibility. The enzymes that are involved in the cascade are separated into three general categories, each of which have distinct roles; E1 ubiquitin activating enzymes (E1s), E2 ubiquitin conjugating enzymes (E2s), and E3 ubiquitin ligases (E3s) (Figure 1.1). E1s take up and functionally activate ubiquitin, allowing it to form a high energy bond with an E2. E2s accept this activated ubiquitin from the E1 and are generally responsible for the catalytic activity of the cascade, conjugating ubiquitin to solvent accessible lysine residues on the substrate. E3s impart substrate specificity, often bridging interactions between the E2 and substrate. This cascade, and the regulation of its individual components, allows the cell to exercise exquisite control over the ubiquitin system. To better understand the dynamics of ubiquitin regulation, I will discuss each of these categories of enzyme.

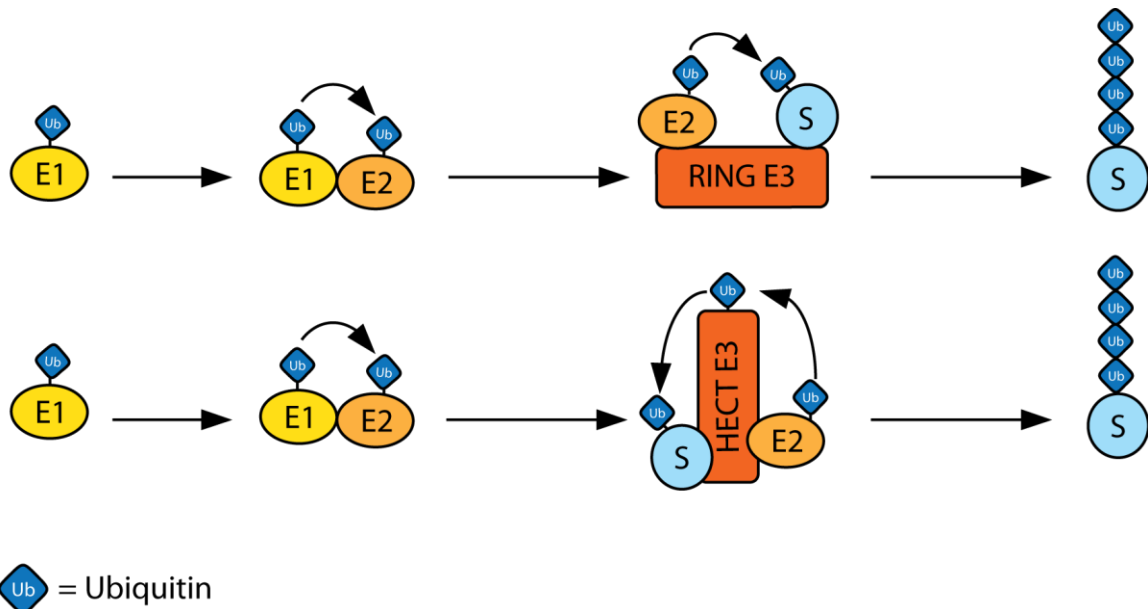


Figure 1.1: The Ubiquitin Cascade

E1s take up free ubiquitin in an ATP dependent manner, then transfer ubiquitin to an E2. E2s then either interact with scaffold-type E3s (such as RING-type E3s) in order to directly conjugate ubiquitin to substrate, or transfer ubiquitin to catalytically active E3s. Catalytically active E3s such as HECT E3s can both directly bind and ubiquitylate substrate. Ubiquitin can also be a substrate of the cascade, resulting in the generation of ubiquitin chains.

E1 Ubiquitin Activating Enzymes

E1s act as gatekeepers for the ubiquitin cascade. Free ubiquitin in the cytoplasm cannot be taken up by E2s, it must first undergo an activation step that is mediated by E1 enzymes, and then is subsequently transferred to the E2. The E1 enzyme noncovalently binds to and acyl-adenylates the C-terminal tail of ubiquitin(3,4). This adenylated residue is then attacked by a cysteine residue in a separate binding face of the E1, which forms a covalent high-energy thioester bond(5). Upon transfer of ubiquitin from the noncovalent binding face to the catalytic cysteine, the E1 continues to take up another ubiquitin protein for adenylation(6,7). This leads to the E1 being loaded with two ubiquitin proteins, one noncovalently associated, and the second covalently bound. The thioester bond formation of ubiquitin with the E1 induces conformational changes that expose an E2 binding site, allowing formation of the E1-E2 complex(8). An E1 that possesses ubiquitin on its second covalent binding face, but is not associated with ubiquitin on its noncovalent binding face, shows reduced kinetics of interaction with E2s, indicating an intrinsic ubiquitin homeostasis sensor in the

first step of the cascade(8). In humans, there are only 2 E1s, Uba1 and Uba6. As E1s must serve as a source of ubiquitin for many structurally diverse E2s, we can infer a level of promiscuity based on the limited number of E1s. Indeed, it has been observed that the entirety of the detectable pool of Uba1, an E1 which functions to provide ubiquitin to the majority of E2s in eukaryotic cells, is in complex with ubiquitin, implying it is constitutively feeding ubiquitin into the system(9). While it has been reported that Uba1 is phosphorylated, and is expressed in distinct isoforms, the functional consequences of these events are not well understood(10,11).

E2 Ubiquitin Conjugating Enzymes

The E2 ubiquitin conjugating enzyme acts as the primary mediator of covalent isopeptide bond formation between ubiquitin and substrate. There are 38 genes for E2 enzymes expressed in humans, and these are generally grouped into four main classes determined by structural homology. Class I E2s are minimal, and most of their sequence is comprised of a core ubiquitin-conjugating domain (Ubc) possessing an active site cysteine residue. Class II E2s have N terminal extensions, Class III E2s have C terminal extensions, and Class IV E2s have both N and C terminal extensions. Perhaps more usefully, the E2s expressed in humans can also be broken into 17 families by phylogenetic relationship, and are denoted by the prefix UBE2 followed by a letter and number, which is indicative of family(12). For all E2s, the Ubc is the element that is recognized by the E1 and ensures a specific E1-E2 interaction(13). The activated E1 binds the Ubc and ubiquitin is transferred from the catalytic cysteine on the E1 to the catalytic cysteine of the Ubc in a transthioylation event. Once the E2 has been charged with ubiquitin, interactions with E3 ubiquitin ligases drive substrate ubiquitylation. A single E2 family can interact with several families of E3, creating a modular system of ubiquitylation in which a core catalytic enzyme can be directed to highly disparate substrates.

All E2s studied to date are recognized by the E1 through an E2 α -helix 1 region in the Ubc, and E2s recognize their E3 counterparts through two loop regions (L1 and L2) and interactions with α -helix 1 (14). This overlapping of binding faces at α -helix 1 indicates that once an E2 has conjugated

ubiquitin to a substrate, it disassociates with the E3 in order to be recharged with ubiquitin by the E1. While the overarching structure of E2s is conserved across family members, variations in the L1 and L2 regions of the E2 provide distinct interaction surfaces. These variations can mediate E2-E3 interactions, allowing the E3 to distinguish between different E2s. Comparing the residues responsible for E2-E3 interaction illustrates this; interaction of UBE2L3 and the E3 Cbl is mediated by completely different residues than the interaction of UBE2D2 and the E3 CNOT, despite involving the same L1/L2/ α -helix 1 regions(15,16).

The variety found in E2 functions not only provide an additional layer of flexibility in regulating ubiquitin system events, but also provides the mechanism by which ubiquitin chain linkage type and length is determined. The specific E2 recruited to a substrate can determine whether a substrate is mono or polyubiquitylated, and what type of polyubiquitin chain is formed(17). Alternatively, E2s can be directed to form specific chain linkages by the E3 with which they interact(18). This functionality can be seen in E2 family Ubch5/UBE2D mediated ubiquitylation. These E2s promiscuously ubiquitylate substrate lysine residues, and have no intrinsic facility for building a specific chain linkage. This provides a common catalytic E2 that can be directed by different E3s in order to form many distinct ubiquitin chain lengths and types(19). It has been seen that some E2s preferentially monoubiquitylate substrate, and that this can serve as an initiating event that promotes polyubiquitylation. After the substrate has been monoubiquitylated, other E2s are recruited and form polyubiquitin chains directly on the initiating ubiquitin molecule(20). The type of linkage these extending E2s form can be a function of the specific E2 that is extending the chain(21). E2 activity is sometimes modulated by interactions with other E2 family members. There is a subfamily of E2s that possess the Ubc, but lack intrinsic catalytic activity(12). These E2s can complex with catalytic E2s, and either serve as activating co-effectors or modulate the catalytic E2 activity(22,23). Additionally, E2s have been shown to be modulated by other cofactors, which can serve not only as recruiting signals but also change the kinetics of ubiquitin transfer(24). These properties allow for a relatively small array of E2s to exhibit a high degree of functional plasticity and regulation.

E3 Ubiquitin Ligases

E3 ubiquitin ligases carry out the final step of the ubiquitin cascade, exponentially increasing the complexity of the ubiquitin cascade. This can be immediately inferred by the sheer number of genes encoding E3s; whereas there are 2 E1s and ~40 E2s, there are over 600 genes encoding E3 ubiquitin ligases in the human genome(25). E3s, through directly interacting with substrate and E2, serve to provide substrate specificity to the ubiquitin cascade.

E3s can be broadly divided into two categories based on functional mechanism: non-catalytic ligases that act as intermediates between an E2 and a substrate, and catalytic ligases that directly conjugate ubiquitin to a substrate (Figure 1.3). The majority of E3s belong to the former class, which are known as RING (Really Interesting New Gene) type E3s. RING-type E3s are defined by a Zn^{2+} binding RING-finger domain or a RING-like domain that mediates their interaction with an E2. These non-catalytic E3 ligases are hugely diverse outside the RING domain itself. Unique combinations of distinct domains lead to highly variable function, impacting substrate binding, E2 interactions, and multimeric complex formation. RING type E3s serve both as scaffolds that promote the interaction of E2 with substrate, and also as adaptors, mediating the ubiquitin chain type formed.

Although almost 95% of E3 ligases are RING-type E3s(25), the first E3 characterized was of another sort entirely. A mammalian E3 known as E6-AP was found to have intrinsic catalytic activity, accepting ubiquitin from the E2, forming a thioester bond and then ubiquitylating substrate directly(5). It was soon seen that the catalytic domain of E6-AP existed in other E3 family members, collecting a group of functionally related E3s into the homologous to E6-AP carboxy-terminal (HECT) domain family(26). We now know that 28 HECT type E3s are encoded by the human genome(5,25). Each of these E3s contains the roughly 350 amino-acid catalytic HECT domain at

their C-terminus, despite varying in size from ~80kDa to up to ~500kDa. While the HECT domain imparts catalytic activity, it is generally regions N-terminal to the HECT that define the substrate specificity of these E3s. Based on shared N-terminal regions found in HECT type family members, human HECT E3s are divided into three subfamilies, the WW domain containing Nedd4 family, the RLD (RCC1-like domain) containing HERC (HECT and RCC1-like domain) family, and a catch-all group of 'other' HECT type ligases which includes the prototypic HECT family member, E6-AP.

Ubiquitin as a variadic, reversible signal.

Ubiquitin itself is a 76-amino acid globular protein that can be conjugated to substrates, most commonly to substrate lysine residues(27). Ubiquitin contains 7 lysine residues (Figure 1.2, *left*). This provides a system in which ubiquitin can be attached to another ubiquitin protein. These linkages can be short or long, homogeneous or heterogeneous. A homogeneous linkage would constitute a chain of ubiquitin molecules attached successively through the same lysine residue, such as a chain formed entirely of lysine 48 linked ubiquitin molecules. Heterogeneous linkages, either through branched chains assemblies or other posttranslational modifications of ubiquitin (such as phosphorylation) are less well studied, but all possible homogeneous linkages have been detected in cells(27,28).

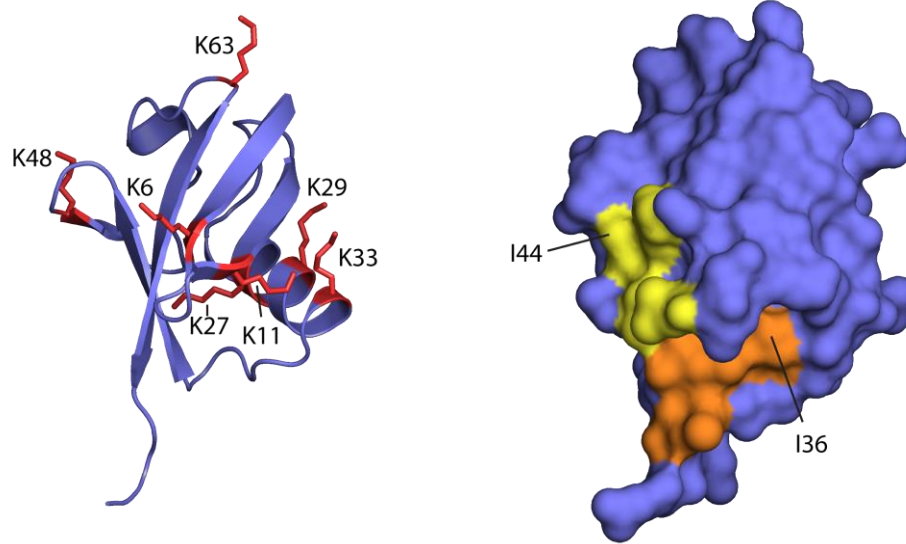


Figure 1.2: Structural features of ubiquitin.

Left: Ribbon cartoon diagram of the structure of ubiquitin. Lysine residues are in red. *Right:* Surface model of the structure of ubiquitin. The I44 hydrophobic patch is in yellow, and the I36 hydrophobic patch is in orange.

binding surfaces of ubiquitin itself, but by the length and orientation of their linking regions(35).

Once information is encoded into these distinct ubiquitin topologies, they must be differentially recognized in order for the information to translate to a functional consequence.

Most commonly the information provided by ubiquitylation is understood through interaction with ubiquitin binding domains, or UBDs. Proteins that contain UBDs are able to translate this information and provide downstream effects(36). It is estimated that there are roughly 150 distinct UBD containing proteins, providing a broad spectrum of downstream functions. Currently, there are 20 recognized families of UBD distinguished both structurally and by type of ubiquitin linkage they recognize. Although most UBDs target the I44 surface of ubiquitin, the amino acids surrounding the I44 patch can vary greatly. Consequently, UBDs recognize a variety of chemical properties proximal to the common I44 patch to decode ubiquitin signals(36). Combining multiple UBDs facilitates a number of functions such as promoting protein complex formations, recognition of specific ubiquitin chains, and increased affinity for ubiquitylated targets(37,38). This provides an

efficient and flexible system in which responsive elements can be attached to domains that recognize specific ubiquitin moieties.

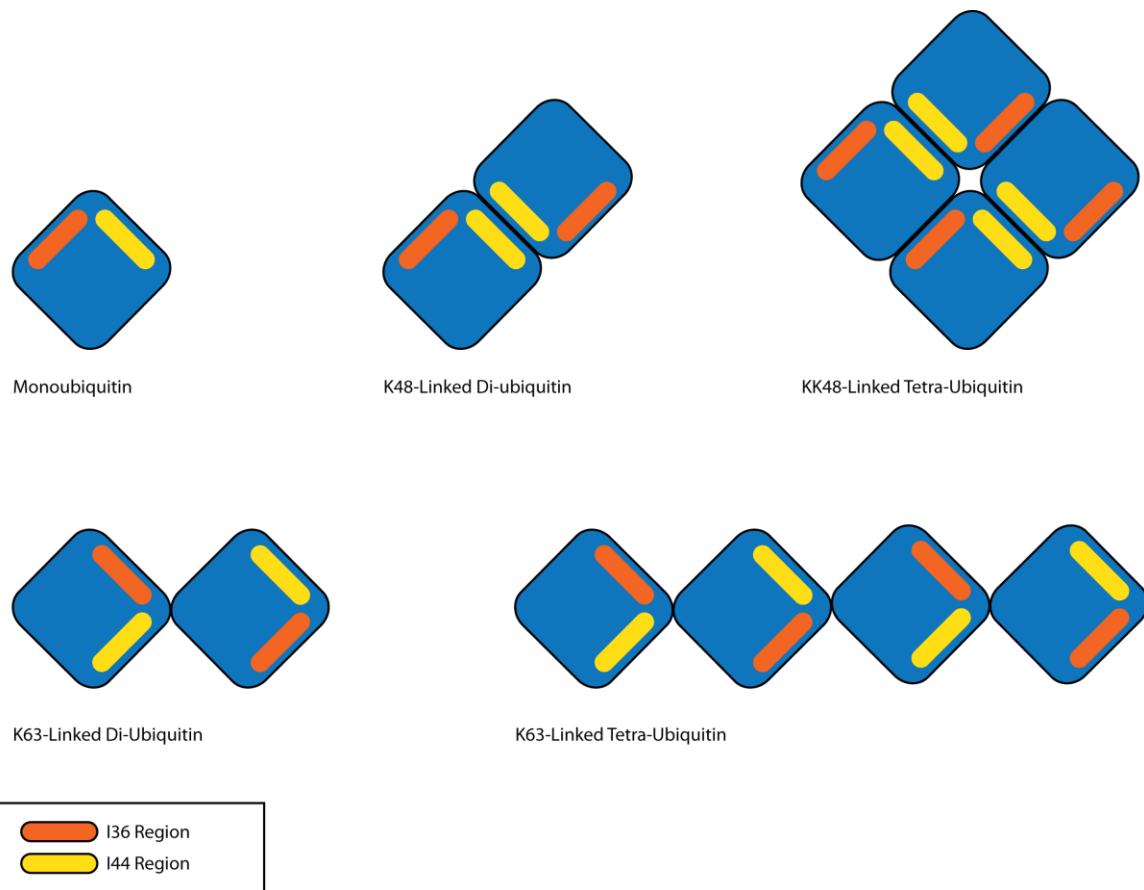


Figure 1.3: Distinct topologies generated by ubiquitin chains.

Examples illustrating how single or multiple ubiquitin linkages can generate a variety of distinct topologies that can be differentiated by detection of specific hydrophobic binding patches. The I36 patch is in orange, and the I44 patch is in yellow, as above.

Deubiquitylating enzymes (DUBs) provide the cell with the ability to edit or delete information conveyed in substrate ubiquitylation. DUBs are able to remove ubiquitin that has been attached to substrates. This can stabilize proteins targeted for degradation, alter protein localization, or allow for re-processing of the substrate(39-42). Additionally, substrates targeted for proteasomal degradation have their ubiquitin chains removed when being processed at the proteasome. This removal allows ubiquitin to be recycled back to the cytoplasm, preserving the ubiquitin pool available in the cell(43). Lastly, it is the action of the DUBs that provides the initial pool of free ubiquitin in the first place. Two of the four genes for ubiquitin encode linear tetramers that must be processed subsequently by the DUB enzymes in order to make the individual ubiquitin proteins available to enter the cascade, maintaining a pool of ubiquitin in the cell(44).

Consequences of ubiquitylation.

This flexibility in encoding information seen in the ubiquitin system allows for a broad spectrum of functional consequences of ubiquitylation. Generally, ubiquitylation is associated with substrate degradation. Degradation of a ubiquitylated substrate can be achieved by two distinct mechanisms: degradation via the proteasome, or degradation via the lysosome. Proteasomal degradation occurs through the ubiquitin-proteasome system (UPS) and is primarily achieved through K48 linked ubiquitylation of substrate(45). The UPS is the major mediator of the degradation of cytoplasmic proteins in the cell, mediating as much as 80-90% of cytoplasmic protein degradation(46,47). The proteasome itself is a protein complex that resides within all eukaryotic cells and is required for cell division(48). These complexes manage the degradation of proteins via proteolysis within a barrel-shaped core particle, known as the 20S particle. Entry into the 20S particle is restricted by the 19 subunit regulatory particle, or the 19S particle(49,50). Proteins recognized by the 19S regulatory particle are unfolded and guided into the 20S core, where they are hydrolyzed into small peptides. The 19S particle also has deubiquitylase activity, allowing it to recognize ubiquitylated substrate and subsequently remove the ubiquitin tag, serving both to recycle ubiquitin into the cytoplasm and restrict substrate degradation(51-54).

Ubiquitin mediated degradation is also a key regulator of membrane-bound proteins and membrane fusion events by promoting receptor internalization and lysosomal degradation. Proteins associated with the plasma membrane are degraded in a ubiquitin-dependent manner(55-59), predominantly by recognition of monoubiquitylated substrate or K63 linked ubiquitin chains(60). In fact, it was once believed that ubiquitin-mediated transport to the lysosome was the primary method by which the cell degraded protein(44). Though it has since been established that the proteasomal degradation system is the major means of degradation for cytoplasmic substrates(47), the lysosomal pathway allows degradation of membrane-bound substrates.

Mechanisms involved in lysosomal degradation provide an excellent model of the functional plasticity of the ubiquitin system. We gain several insights into how ubiquitin sorting is utilized in

the lysosomal pathway by through the related multi-vesicular body (MVB) sorting pathway in yeast. Mono-ubiquitylation of plasma membrane associated proteins and receptors mediates their internalization and sorting into the endosomal pathway(40,44,61). However, in order for some proteins to be targeted to the MVB (and thus the vacuole in which they will be degraded), monoubiquitylation is not sufficient. It is not until these internalized proteins are modified with K63 linked ubiquitin chains that they are sorted to the MVB(44). This same mechanism serves to direct membrane-associated proteins from the Golgi directly to the MVB, bypassing the plasma membrane entirely(55). This sorting system is at least partially preserved in eukaryotes, and we can identify these ubiquitin sorting events in mammalian cells. Interestingly, some receptors appear to only require multi-monoubiquitylation events in order to be internalized and directed to the lysosome(58), whereas others require K63-linked polyubiquitylation(62). Despite the predominance of distinct ubiquitin linkages in the two proteolytic pathways, recognition of these linkages as degradation markers is contextual. K63 linkages have also been implicated in non-proteolytic pathways, indicating that additional inputs are needed for deterministic signaling events(63,64).

Aside from degradation, ubiquitin also regulates other cellular functions. Substrate localization, enzyme activation, and protein-protein interactions can all require ubiquitin. The diverse array of ubiquitin binding domains encoded into proteins allows ubiquitin to be recognized by effector proteins and promote interaction. Mono-ubiquitylation and K63 polyubiquitylation of a protein can often promote recruitment of interacting factors to form macromolecular complexes in response to specific events(65,66). These complexes can then be disassembled when deubiquitylating enzymes remove the ubiquitin molecule, terminating the protein interactions(67). Protein-protein interactions can also be inhibited through ubiquitylation – they have been seen to block interaction sites or promote intramolecular interactions that result in conformational changes that abrogate interaction with effector molecules(68). With such diverse consequences, specific ubiquitylation events are under tight control. As E3s are the ultimate determinant of substrate specificity, they are an important target of regulation.

Regulation of E3 ligases

RING-type ligases

There are repeating themes in the regulation of the components of the ubiquitin cascade. Two mechanisms common to E3 ligase regulation are that of substrate recruitment and E2 binding. We can observe these two mechanisms regulating the multi-subunit RING E3 ligase CRL family. Each CRL utilizes a Cullin-family scaffold on which it assembles an E2-recruiting RING-domain protein at the C-terminus and an interchangeable adaptor protein at the N-terminus. This scaffold protein either has intrinsic substrate binding capacity or, commonly, associates with a substrate-binding factor. This interchangeable architecture allows a common CRL platform to quickly change the array of substrates it interacts with and to selectively recruit an E2. This is perhaps best exemplified in the case of the Cul1 family of CRLs, or SCF (Skp1-Cul1-Fbox) ligases. Within the SCF, Cul1 serves as the Cullin scaffold protein, Rbx1 as the RING-type E2 recruitment domain, and Skp1 as the adaptor that recruits the substrate specificity factor Fbox. The flexibility of the SCF E3s relies on the interchangeable Fbox protein; 69 Fbox proteins have been identified in humans(69). Utilizing this array of Fbox substrate selection factors, the Skp1-Cul1-Rbx1 core has been found to be a key factor in an array of cellular processes, including apoptosis, proliferation, mitosis, cell cycle regulation and DNA damage repair(21,70-72).

One notable regulatory characteristic observed in many RING type E3s is that they can function as monomers, dimers or oligomers. The mono- or multi-meric state of RING ligases can play a regulatory role, and thus define the role of a particular RING E3 in context. Heterodimerization can exert a protective influence on an E3, negatively regulating its ability to promote its own ubiquitylation and thus protecting it from degradation(73). Conversely, homodimers of E3s have been seen to drive their own ubiquitylation with increased kinetics and mark themselves for degradation(74). Thus, through blocking dimerization, E3s can be both positively and negatively regulated(75-78).

HECT-type ligases

HECT type ligases present an interesting regulatory problem relative to other E3s. Functionally, once charged, they are essentially E2-E3 hybrids. This means that not only are they capable of recruiting substrate, they are also primed for activity and the ultimate determinants of ubiquitin chain type specificity(79). HECT type family members, despite consisting of less than 5% of the total unique E3 ligase family members, have been shown to regulate a variety of cellular processes and exhibit the ability to synthesize each type of ubiquitin chain(80). The chain specificity of HECT type ligases appears to be mediated by a 60 amino acid region at the far C-terminus(79). The prototypic HECT E3 in yeast, Rsp5, preferentially forms K63-linked ubiquitin chains(79). Rsp5 mediated K63-linked ubiquitin chain formation has been described as a regulator of several membrane trafficking events(81-84). Conversely, the mammalian HECT type E3 E6-AP modifies substrate with K48 linked ubiquitin chains, which traditionally target substrate for the proteasome(79). There are cofactors that, when bound to Rsp5, promote Rsp5 mediated mono-ubiquitylation of substrate, indicating that chain specificity of HECT type family members can be modified(24). Additionally, like RING E3s, some HECT type family members form multi-meric complexes that can modify the kinetics of substrate ubiquitylation or alter substrate specificity(85-87). Thus, we see the reappearance of regulatory themes from both E2 (modification of ubiquitylation type and kinetics) and RING E3 mechanisms (E2 recruitment, substrate specificity) providing a highly dynamic and robust family of ubiquitin ligases.

Intrinsic catalytic activity distinguishes a multifunctional family of E3 ligases.

The ability to bind substrate and directly catalyze the addition of ubiquitin implies that these E3s can be effectively primed for activity, and must be tightly regulated to ensure appropriate signaling. One important characteristic shared by many HECT E3s that addresses this is autoinhibition, which allows these E3s to deploy in the cell at concentrations and subcellular locations that allow them to be immediately effective. This spatial and temporal property makes these E3s particularly useful in

directing a variety of substrate fates. This is perhaps best exemplified in the Nedd4 family of HECT E3 ligases.

Nedd4 E3 ligases illustrate several mechanisms of regulatory control, from substrate recruitment to activation. Of the two structurally defined HECT families, the Nedd4 family of E3 ligases is the largest, comprised of 9 family members(80,88,89). Indicative of its biological significance, the architecture characteristic of Nedd4 family members has remained largely conserved in eukaryotes, tracing its origins to the essential HECT E3 Rsp5 in yeast(90). All Nedd4 family members possess an N-terminal C2 (calcium-dependent lipid binding) domain, 2-4 WW (protein-protein interaction) domains, and the characteristic HECT domain on the C-terminus (Figure 1.4). Despite the high degree of homology among Nedd4 family members, and their relatively small presence as a portion of all E3 ligases, these HECT type E3s have been found to be key regulators of a diverse array of physiological functions, and Nedd4 family members have been implicated in neuronal development, cancer, and immune function(41,91-98). In multiple Nedd4 family members, the C2 domain is involved in an intramolecular interaction that regulates autoinhibition of the catalytic activity of the HECT domain(99-102). Relief of autoinhibition via calcium signaling, phosphorylation, or adapter interactions promotes the catalytic activity of many Nedd4 E3s(100,101,103-105). This intrinsic molecular 'brake' helps to ensure that these E3s are only activated in the correct context.

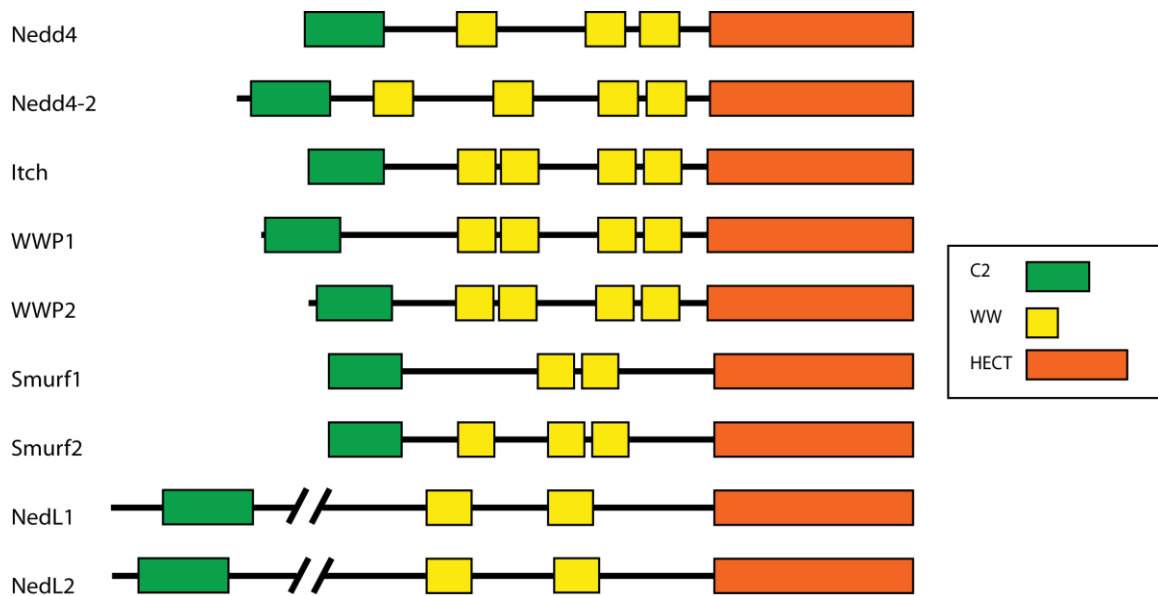


Figure 1.4: The Nedd4 family of E3 ligases possesses a conserved, modular architecture.

Cartoon representations of the Nedd4 family of HECT E3 ligases, aligned. The N-terminal calcium and phospholipid binding C2 domain is in green. The WW domains, which bind PY and poly-proline motifs to recruit substrates, are in yellow. The C-terminal catalytic HECT domain, which binds the E2, accepts ubiquitin and conjugates ubiquitin to substrate, is in orange.

To understand the regulation of Nedd4 family regulated signaling networks, an appreciation of the modular nature of Nedd4 architecture is needed. While it is possible to evolve novel domains, overwhelmingly new function is formed by varying the combinations of existing domains(106,107). With this in mind, we can consider proteins as an assemblage of smaller units, each of which contributes distinct attributes to the action of the whole. These modules confer a multitude of abilities, from enabling a protein to localize to a specific subcellular compartment to specifying the pathway in which it will be active. For example, the SH2 (Src homology 2) domain is included in several cellular signaling molecules, and confers to these signaling molecules the ability to recognize and bind to sequence-specific phosphotyrosine residues(108). Therefore, we can understand that a protein that possesses an SH2 domain may be sensitive to tyrosine phosphorylation, and is likely a component of regulatory networks in which we see tyrosine kinases. By utilizing an array of modular combinations, the cell can develop the regulatory networks necessary to detect and integrate information in a complex environment of internal and external cues. To appreciate the potential functions of a protein, a thorough understanding of its domain composition is informative.

Contributions of the HECT domain to Nedd4 family function.

The most well conserved domain sequence in the Nedd4 family, the catalytic HECT domain is an intriguing element when considered in a regulatory context. While it is generally thought that the structure of the HECT domain determines the type of ubiquitin chains it synthesizes, there are conflicting results regarding exactly which type of ubiquitin chain each Nedd4 family member builds, and at what length(41,79,109,110). The HECT domain itself is comprised of an 'N-lobe', in which resides the E2-binding region, and a 'C-lobe' which houses the catalytic cysteine residue that accepts ubiquitin from the E2 through a thioester bond. The two lobes are connected by a flexible hinge region, and display a high degree of conformational flexibility. HECT type E3 ligases both mono- and poly-ubiquitylate substrate, and form K63, K48, K33, K11, and K29 linked chains(79). It is thought that the HECT domain is structurally constrained to generate specific polyubiquitin chain linkages, and whether this can be modulated is unknown. While Nedd4 family members have been shown to be regulated at the level of E2 – HECT domain recruitment and interaction, whether there are functional consequences to specific E2 interactions has gone largely unexplored.

Contributions of the C2 domain to Nedd4 family member function.

The conservation of the N-terminal C2 and WW domains among the Nedd4 family members is not only striking, it is informative. As mentioned previously, all Nedd4 family members have an N-terminal C2 domain, 2-4 WW domains and a C-terminal HECT domain. The C2 domain is a classical mediator of vesicular trafficking, membrane fusion, and signal transduction events in the cell. Evolutionarily, C2s first appear in eukaryotic organisms, and the Nedd4 family C2 is most closely related to the PKC-C2 family of C2s. C2s were first identified as a calcium-dependent membrane interaction region of the PKC family(111). This family of C2 domains exhibits both calcium binding properties and phospholipid interactions(112). C2 domains often act as Ca^{2+} activated membrane-association modules. PKC-C2 family C2s have 2-3 calcium binding regions, and binding to calcium through these regions mediates interaction with phospholipids. Calcium acts as both an electrostatic switch, favoring lipid interactions, and as a functional bridge between the

C2 domain and the phospholipid(113). In general, PKC-C2 family members often bind to phosphatidylserine(111). However, PKC-C2s also possess a second phospholipid binding lysine-rich cluster. This lysine-rich cluster has been shown to bind phospholipids in a calcium independent manner, and is generally associated with binding to phosphoinositides(114-117). This binding site has been shown to mediate interactions with SNARE proteins and AP2 and be important in clathrin-mediated endocytosis at the plasma membrane(117,118), once again reinforcing the concept of the C2 domain as a membrane localization signal.

We see these same membrane interaction behaviors preserved in many Nedd4 family members. The yeast homologue of Nedd4, Rsp5, binds a broad array of phosphoinositides in a calcium independent manner(81). Interestingly, while the Rsp5-C2 was shown to bind all phosphorylated phosphatidylinositols, it did not bind phosphatidylserine or phosphatidylcholine, which reflects the calcium-independent binding characteristics of other PKC-C2 family members(81). This binding activity was critical for the ability of Rsp5 to mediate the endosomal sorting of cargo from the trans-Golgi network to the vacuolar lumen. Nedd4 family members have also been shown to exhibit the calcium-dependent binding characteristics of PKC-C2 family members. *In vitro* assays showed that the Nedd4 C2 bound to both phosphatidylserine and phosphatidylcholine in a calcium dependent manner(119). While this is intriguing, the biological relevance of phosphoinositol interaction with the C2 domain of Nedd4 family members is not well understood. While it has been shown that mutants lacking a C2 domain no longer traffic to membranes in response to calcium signaling, whether this is due to a direct interaction with phospholipids or requires a cofactor is unclear(119).

Despite these observations, the general importance of the C2 domain in regulating membrane interactions has not been observed across Nedd4 family members. However, the C2 domain has been established as a site of substrate recruitment in several Nedd4 family members. Rsp5 regulates vesicular trafficking through C2 interactions with both membrane lipids and by interacting with other effectors of vesicle trafficking(120). Cells can utilize this function of the C2 domain in

very straightforward ways; for example, alternative splicing of Nedd4 family members can result in isoforms lacking C2 domains. Nedd4L, the closest homologue to Nedd4, has been observed in three distinct isoforms, one of which lacks the C2 domain. This isoform is not capable of cycling from the plasma membrane to the endosome in response to calcium, whereas Nedd4L isoforms containing the C2 domain display this ability. Additionally, the C2-deficient isoform is defective in epithelial sodium channel regulation, a known substrate of Nedd4L(121).

Underlining the importance of a contextual understanding of regulatory elements, activity of the Nedd4 family member Smurf1 is highly dependent on C2-mediated localization. Smurf1 has been implicated in a variety of substrate interactions, and the C2 domain has been implicated not only in localizing Smurf1 to the appropriate compartment but also direct interactions with substrate. Full length Smurf1 localizes to the plasma membrane in a C2 dependent manner. Truncation mutants that lack a C2 domain were unable to localize to the plasma membrane, and instead were found diffusely throughout the cytoplasm(122). Mutation of residues in the C2 region associated with phospholipid binding resulted in a similar cytoplasmic dispersion of Smurf1, indicating that not only is the C2 domain important, but it's phospholipid binding region plays a role(122). When Smurf1 was unable to localize to the plasma membrane, its action on established substrates shifted substantially, indicating that Nedd4 family members not only interact with substrate through distinct mechanisms but that it is possible to selectively attenuate these interactions by targeting regulatory modules(122,123).

A major question that remains is the significance of the C2 domain in the Nedd4 family members Itch, WWP1, and WWP2. These Nedd4 family members are critical regulators of a variety of cell functions, mediating events from apoptosis to iron homeostasis. Each of these possesses a C2 domain, but its physiological relevance remains unclear. Research investigating activity of truncation mutants of Itch indicates that unlike other Nedd4 family members, there appears to be no role for the C2 domain in mediating autoinhibition or recruitment to the plasma membrane(119,124-129).

Contributions of the WW domains to Nedd4 family member function.

The WW domains of Nedd4 family members are traditionally associated with substrate recruitment(130-132). This narrow interpretation does not convey the full significance of WW domains, as it ignores a key feature of WW domains. Multiple WW domain repeats impact substrate interactions, and tandem WW domains are capable of intramolecular interactions(133,134). WW domains are among the smallest conserved domains seen in biology, consisting of a stretch of 35-40 amino acids that form a small globular domain(135). Each WW domain consists of a three stranded beta-sheet fold that is characterized by its ability to interact with polyproline motifs. Generally, WW domains are divided into four classes based on their ability to interact with different peptide motifs(136). Class I WW domains predominantly bind to PPXY motifs, Class 2 to PPPL/R motifs, Class 3 to (PxxGMxPP)*2 motifs, and Class4 WW domains to phosphoserine-proline or phosphothreonine-proline residues. There are over 100 WW domains and nearly 2,000 Class I binding motifs found in human proteins(137). Each of these classes is generally found to be promiscuous when analyzed in isolation, and they display the capacity to cross-interact with motifs of other classes with varied affinities(130). While this facet could be considered a caveat for identifying substrates, it would be better considered as an inherent feature of WW domains that must be accounted for. Weak affinities for substrates can be overcome through multiple WW domain – binding motif interactions, and the availability of these motifs for binding should be investigated when considering substrate interactions.

WW domains commonly facilitate oppositional regulation in signaling pathways, in which one WW-domain containing protein exerts a positive influence and another a negative on a common substrate. While displaying a high degree of cross-reactivity, small differences in the amino acid sequence of a WW domain can modulate affinities for specific motifs(133). This allows WW domain containing proteins to competitively bind to substrates, as can be seen in the regulation of the receptor tyrosine kinase ErbB4 by either WWOX or YAP. The WW domain containing protein YAP is a positive regulator of ErbB4 activation and, through this, cell proliferation(138). Increased

concentrations of WWOX (WW domain containing oxoreductase) bind ErbB4 preferentially and sequester it to the cytoplasm, inhibiting proliferation(139).

Both the number of WW domains and their relative positioning are important when considering their function in Nedd4 family members. Tandem WW domains are a feature commonly seen in WW domain containing proteins, and different combinations of WW domain repeats can impart altered behaviors. For example, WWOX interaction with ErbB4 requires both WW domains 1 and 2 of WWOX(138). However, the direct interaction of WWOX with ErbB4 is mediated by WWOX's N terminal WW domain (WW1). The second domain of WWOX, however, exhibits no direct binding to ErbB4 or PY motifs. This WW domain instead mediates PPxY motif binding by acting as an intramolecular chaperone, enhancing the stability of WW1 and increasing its affinity for PPxY motifs of multiple substrates up to threefold(138,139). Nedd4 family member WW domain interactions with substrate are dynamically modified by phosphorylation events in several ways. Phosphorylation of both substrates and the WW domains that bind them can either enhance or inhibit interactions in a generally predictable manner. Phosphorylation of substrates can be actively manipulated to alter substrate-WW domain affinity and tip the balance of regulation either in favor of or against a Nedd4 family member. Importantly, we see that specific WW domain residues that are only partially conserved appear to mediate binding to pS/pT motifs. We see this most evidently in the coordination of the regulatory elements in the Smad signaling pathway. Phosphorylation of serine and threonine residues on Smad2/3 by Cdk8/9 recruits the activating cofactor Pin1 via a Class IV WW domain – pS/pT interaction(140,141). However, phosphorylation of a neighboring PPxY motif by GSK increases the affinity of the WW3 domain of Nedd4L to the PPxY motif. The recruitment of the WW3 domain of Nedd4L functionally concentrates the WW2 domain of Nedd4L at the pS/pT region preferentially bound by Pin1, leading to Pin1 eventually being outcompeted by Nedd4L and the subsequent degradation of Smad2/3(142,143). Smurf2 has also been seen to operate in a similar manner, but with some interesting nuances. Both Cdk8/9 and GSK phosphorylate serine and threonine residues residing in a proline rich region of Smad2/3. GSK phosphorylation of Smads is specific to a location only 8 amino acids away from those that Cdk8/9 phosphorylate. This shift

positions the phosphorylated residues closer to the hydrophobic binding pocket of the WW domain, increasing Smurf affinity for Smad (141,143). While the WW domains of Nedd4L involved in interaction with Smad2/3 are generally well conserved across all Nedd4 family members, the pS/pT interacting residues in the WW1 domain of Smurf1 appear specific to the Smurf subfamily. It is worth noting that these residues align well with that of Yap2's second WW domain, a known Smad activating factor. The only known isoform of Yap specifically lacks this WW domain, indicating the role of isoform expression in modulating target specificity.

The Nedd4 family in action.

The differential functions of Nedd4 family members are well illustrated when identifying their roles in regulating lymphocytes. When stimulated, lymphocytes must develop from small cells at rest to highly proliferative secretory cells(144,145). In order to mediate immune responses, lymphocytes must integrate a complex environment of growth, proliferation, differentiation and death signals(146-148). Nedd4 family members can be found as regulators in signaling pathways in each of these processes. The prototypic Nedd4, for example, is a regulator of Insulin-like growth factor-1 (IGF-1) receptor internalization, which is known to be important in T effector cell differentiation, proliferation and survival(149-152). Nedd4 is also an important mediator of T effector cell sensitivity to co-stimulation, promoting degradation of the RING-type ligase Cbl-b, a suppressor of CD28 signaling(153). The Nedd4 family member Itch is extensively involved in immune cell regulation as both a suppressor of cytokine signaling and apoptosis. Perhaps the most well studied of the Nedd4 family members in an immunological context, Itch regulates AP-1 mediated cytokine signaling and cell cycle control, c-FLIP and Numb mediated apoptotic signaling, and NF κ B mediated inflammatory signaling(41,154-158). Finally, while the role of the Smad ubiquitylation regulatory factors 1 and 2 (Smurf 1 and 2) in lymphocyte function is not entirely clear, both Smurf1 and Smurf2 have been shown to be critical to the mediation of TGF-B signaling through regulation of Smad2/3 and inhibitory Smad7 signaling(122,141,159-163). However, while it is generally understood that these Nedd4 family members are capable of acting on several levels of immune cell function, it

remains unclear how these highly homologous enzymes are capable of such distinct action. In order to investigate the capacity for differential function of these Nedd4 family members, a clearer understanding of the mechanisms involved in activation and inhibition is necessary. Additionally, because these Nedd4 family members possess this high degree of structural similarity, while simultaneously maintaining distinct roles in cell signaling, understanding the molecular mechanisms that regulate Nedd4 family members can provide potential tools for specific and appropriate targeting of therapeutic approaches. Therefore, this dissertation describes the overarching structural characteristics and regulatory mechanisms that differentiate subsets of Nedd4 family members. Chapter 2 describes molecular mechanisms regulating inhibition and activation of the Nedd4 family member Itch and a subfamily of related E3s, and how adaptor proteins interact with these E3s through multiple PY motifs in order to activate them. Chapter 3 discusses the results described in Chapter 2 in a broader context of both signaling pathways and their physiological relevance.

Chapter 2 - Itch WW domains inhibit its E3 ubiquitin ligase activity by blocking E2-E3 transthiolation¹

Abstract

Nedd4-family E3 ubiquitin ligases regulate an array of biologic processes. Autoinhibition maintains these catalytic ligases in an inactive state through several mechanisms. However, while some Nedd4-family members are activated by binding to Ndfip proteins, how binding activates E3 function remains unclear. Our data reveals how these two regulatory processes are functionally linked. In the absence of Ndfip1, the Nedd4-family member Itch can bind an E2 but cannot accept ubiquitin onto its catalytic cysteine. This is because Itch is autoinhibited by an intramolecular interaction between its HECT and two central WW domains. Ndfip1 binds these WW domains to release the HECT, allowing transthiolation and Itch catalytic activity. This molecular switch also regulates the closely related family member WWP2. Importantly, multiple PY motifs are required for Ndfip1 to activate Itch, functionally distinguishing Ndfips from single PY containing substrates. These data establish a novel mechanism for control of the function of a subfamily of Nedd4 E3 ligases at the level of E2-E3 transthiolation.

Introduction

Catalytic HECT E3 ubiquitin ligases provide substrate specificity to the ubiquitylation cascade and mediate the transfer of ubiquitin to substrate. The largest family of HECT ligases, the Nedd4-family E3 ubiquitin ligases, regulates signaling in intracellular pathways that control cancer, blood pressure, iron metabolism, and inflammation(41,91-98). Nedd4-family E3 ubiquitin ligases are catalytic E3s that share a conserved modular architecture(90). This includes an N-terminal C2 domain, 2-4 WW domains, and a C-terminal homology to E6AP carboxy terminus (HECT) domain that is the defining feature of HECT E3 ligases. The catalytic HECT-domain is comprised of an E2

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binding “N-lobe” and a catalytic cysteine residue in the “C-lobe”. The catalytic cysteine accepts ubiquitin from an E2 in a transthioylation reaction to form a HECT~Ub intermediate, rendering the ligase catalytically active(164). WW domains of Nedd4-family E3s bind several proline rich motifs, including L/PPxY motifs (x denotes any amino acid), and thus facilitate binding of Itch to substrates(132,165).

The catalytic function of Nedd4 E3 ligases is restrained through autoinhibition. The majority of Nedd4 family members appear to be autoinhibited by an intramolecular interaction between the N terminal C2 domain and the HECT domain(101). Supporting this, removing the C2 domain from Nedd4 and Rsp5, a Nedd4-family orthologue found in yeast, releases autoinhibition. Additionally, phosphorylation can promote the activation of Itch, Nedd4, and Nedd4-2(104,166,167). Among Nedd4-family members, Smurf2 is of particular interest as it not only shows C2 domain autoinhibition, but is further regulated by a reduced affinity binding site for its complementary E2s, necessitating its interaction with the adaptor Smad7 for full function(99,161). Recent work has provided insight into how the C2 domain mediates autoinhibition of both Nedd4 and Smurf2. Specifically, the C2 domain interaction with the HECT domain maintains the ligase in a low activity state, in which the HECT domain has reduced capacity for noncovalent binding of ubiquitin(101).

Adaptor-ligase interactions can also promote Nedd4 family catalytic activity. Indeed, such interactions are known to promote the function of the sole Nedd4-family member in yeast, Rsp5(82,168-170), suggesting that this means of controlling Nedd4-family function is evolutionarily conserved. One adaptor that promotes Rsp5 function is Bsd2, the orthologue of mammalian Nedd4-family interacting proteins (Ndfips). The polyubiquitylation activity of several Nedd4-family E3 ligases is promoted by Ndfips(93,105). These proteins have three transmembrane spanning regions and three L/PPxY (PY) motifs, the motifs recognized by WW domains. Ndfip1 has been shown to promote the ubiquitylation activity of several Nedd4-family members *in vivo*, including Itch, Nedd4-2 and WWP2(92,93,105). As with many proteins that increase the function of Nedd4-

family members, these proteins are commonly referred to as adaptors, but how they promote E3 function is often not known.

The Nedd4 family member Itch plays a critical role in the immune system, with known roles in hematopoietic cells, innate immune cells, and the differentiation and function of T helper cells(41,93,171). In CD4+ T cells, loss of Itch leads to increased T_H2 polarization *in vivo* and *in vitro*, a phenomenon attributed to the increased stability of an Itch substrate, the IL-4 transcription factor JunB(171). Humans with Itch loss of function mutations also exhibit pleiotropic immune dysfunction(172). Strikingly, loss of Ndfip1 also leads to increased T_H2 skewing controlled, in part, by accumulation of the Itch substrate JunB(93,97). *In vitro* Itch is autoinhibited(104,105). It has been shown that phosphorylation of Itch by JNK1 can promote Itch activity(104). Additionally, Itch can be activated by its interaction with Ndfip1(105). The dependence of Itch on Ndfip1 for function suggests that phosphorylation alone is not sufficient to promote Itch function. How intramolecular autoinhibition prevents Itch catalytic function, and how Ndfip1 binding releases this, has yet to be determined.

Here, I have defined the enzymatic step at which Itch catalytic function is inhibited. Itch autoinhibition is maintained by an intramolecular interaction between its HECT and two core WW domains. This autoinhibited state prevents E2-Itch transthiolation from occurring, thereby rendering Itch catalytically inactive. Ndfip1 binding to Itch releases the autoinhibitory conformation that holds Itch in an 'off' state. Binding of Ndfip1 to the core WW domains releases the HECT and allows the transthiolation reaction between the E2 and Itch to take place. My data show that these regulatory mechanisms may be true for WWP2 and possibly other Nedd4-family members.

Results

Itch catalytic function is restrained by a block in E2-E3 transthiolation that is independent of E2 binding.

Itch has been shown to be autoinhibited⁽¹⁰⁵⁾ in a way that can be relieved by interaction with Ndfip1, but the stage of the catalytic cycle at which Itch is autoinhibited is unknown. Thus, I first asked whether Ndfip1 was required for E2-E3 transthiolation, a requisite step in Itch E3 activation. I incubated Itch, in the presence or absence of Ndfip1, with ubiquitin loaded E2 (UbcH7). To track ubiquitin transfer to Itch, I used biotinylated ubiquitin and visualized using streptavidin. No ubiquitin was transferred to Itch in the absence of Ndfip1 (Figure 2.1A), however, when the cytoplasmic region of Ndfip1 was added to the reaction, transfer of ubiquitin to Itch became evident. This was confirmed to be a transfer of ubiquitin to the catalytic cysteine of Itch, and not autoubiquitylation, by demonstrating that the Itch~Ub species was lost after DTT treatment (Figure 2.1B). This confirmed that Itch is autoinhibited at a catalytic step prior to ubiquitin ligation or interaction with substrate.

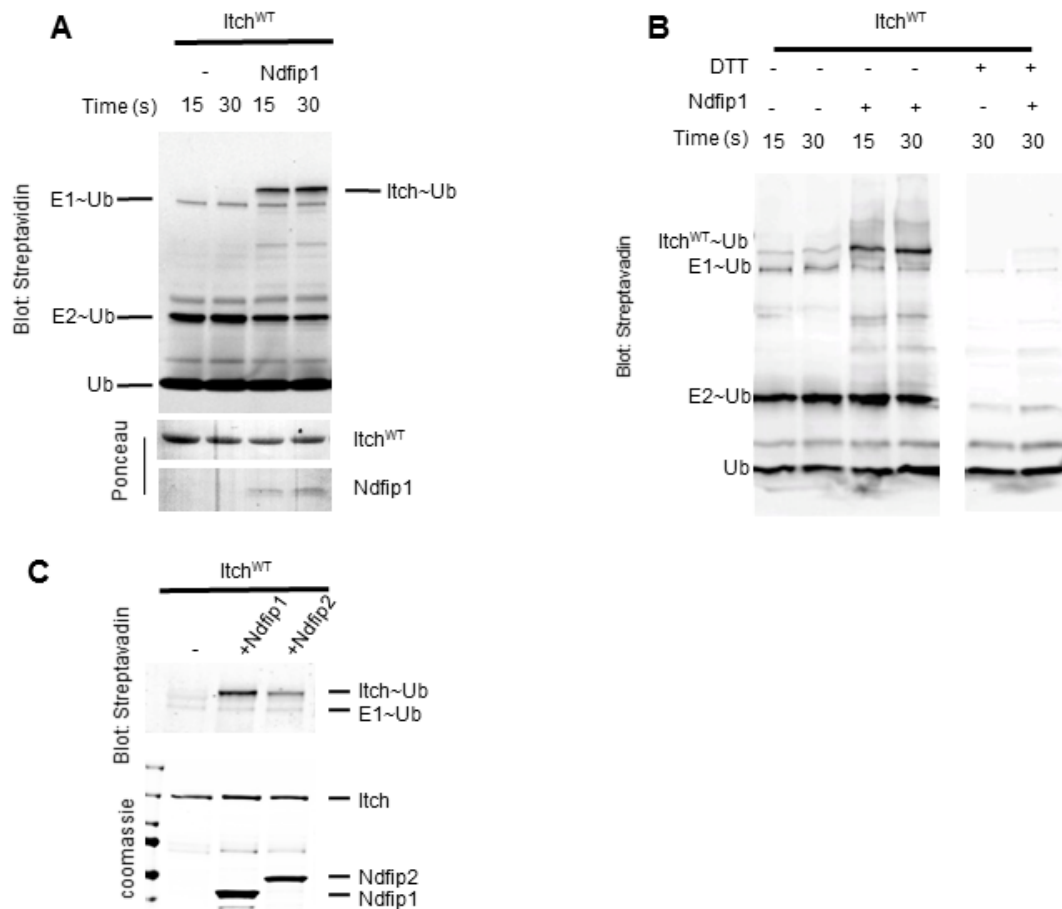


Figure 2.1: Ndfip1 promotes ubiquitin charging of Itch.

A. Itch was incubated for the indicated time points with (biotinylated) ubiquitin loaded E2 and in the presence or absence of Ndfip1. Streptavidin blotting revealed ubiquitin charged Itch. Ponceau staining was used for loading control. B. Samples from the Itch charging experiment shown in Fig. 1A were divided into two tubes and one tube was treated with 50 mM DTT. DTT treatment resulted in loss of ubiquitin charging on Itch, indicating the interaction was mediated by the characteristic thioester bond between the C-terminal glycine tail of ubiquitin and the catalytic cysteine in the HECT domain of Itch. For all panels, similar results were obtained in at least 3 independent experiments. C. Itch was incubated for 30 seconds with biotinylated ubiquitin loaded E2, adding either GST alone, GST-Ndfip1, or GST-Ndfip2. Streptavidin blotting revealed ubiquitin charged Itch species, as above. Coomassie staining was used for loading control.

This block in Itch ubiquitin charging suggested that Itch might be unable to bind to its conjugate E2 in the absence of an adaptor, as has been shown with Smurf2. To test this, I examined Itch-E2 binding in the presence or absence of Ndfip1. Native gel analysis of Itch/E2 complexes showed that Itch does not require Ndfip1 to associate with an E2 (Figure 2.2A). To further test whether autoinhibited Itch could interact with its E2, I compared the E2 binding of full length Itch to that of the Itch HECT domain (Itch^{HECT}) alone, which is robustly active in the absence of Ndfip1. I tested

the binding of full length Itch (Itch^{WT}) and the Itch^{HECT} to Ubch7 as well as Ubch5b (Figure 2.2B,C). Interestingly, Itch^{WT} showed a marked preference for binding Ubch5B in this assay. However, it remains to be seen whether these data imply any functional preference of Itch for an E2 encountered *in vivo*. Nevertheless, these data show that full length Itch can bind an E2 with similar affinity to that of the HECT domain alone, indicating that Itch can interact with an E2 even in its autoinhibited state. Therefore, unlike Smurf2, Itch autoinhibition does not appear to be controlled at the level of E2 interaction. This fits well with structural data showing that, unlike Smurf2, Itch does not have a suboptimal E2 binding pocket(161).

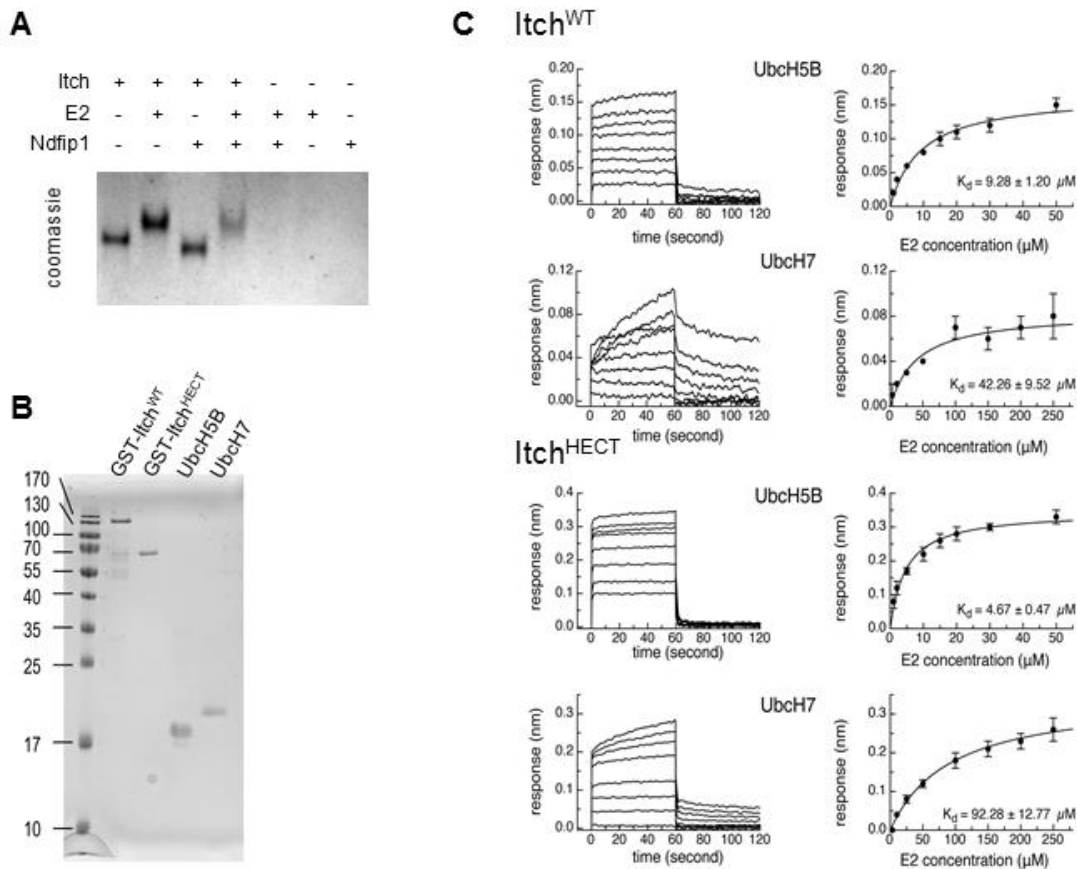


Figure 2.2: Itch interacts with an E2 in the absence of Ndfip1.

Itch / E2 complexes were analyzed by coomassie under native conditions in the presence or absence of Ndfip1. The migration of Itch is altered upon inclusion of the E2 Ubch5b, indicating that Itch interacts with an E2 in the absence of Ndfip1. B. Characterization of purified protein by SDS-PAGE. Itch, Itch^{HECT}, Ubch5B and Ubch7 were recombinantly expressed and purified, then analyzed by SDS-PAGE. C. Itch/Ubch7 and Itch/Ubch5b binding kinetics were analyzed by Octet Biolayer Interferometry. The K_d of Ubch5B and Ubch7 for Itch^{HECT}, known to be catalytically functional *in vitro*, was compared to their K_d for Itch^{WT}. The K_d of the E2 interaction with Itch^{WT} indicate that this is a functionally relevant interaction.

Itch autoinhibition is mediated by HECT and WW domain interactions.

One motif proposed to maintain Itch autoinhibition lies in a proline rich region unique to Itch, between the C2 and its most N-terminal WW domain(104,105). To test the contribution of this region to the block in E2-E3 transthiolation, I generated a truncated version of Itch (Itch^{WW1-HECT}, illustrated in Figure 2.3A). Surprisingly, as with Itch^{WT}, ubiquitin transfer to Itch^{WW1-HECT} was promoted by, and thus dependent upon Ndfip1 (Figure 2.3A,B). Thus, even in the absence of the

enzyme (Figure 2.4B), supporting that WW domains block transthiolation of the catalytic cysteine in the HECT domain. To directly test the importance of the Itch WW domains in autoinhibition, I introduced point mutations in each of the four WW domains of the full length Itch construct (Itch^{WW1-4A} illustrated in Figure 2.4C). These four point mutations were sufficient to relieve autoinhibition and allow ubiquitin charging of Itch (Figure 2.4D). Addition of Ndfip1 to this reaction had no effect. Thus, Itch autoinhibition is mediated by an intramolecular interaction between its HECT and WW domains.

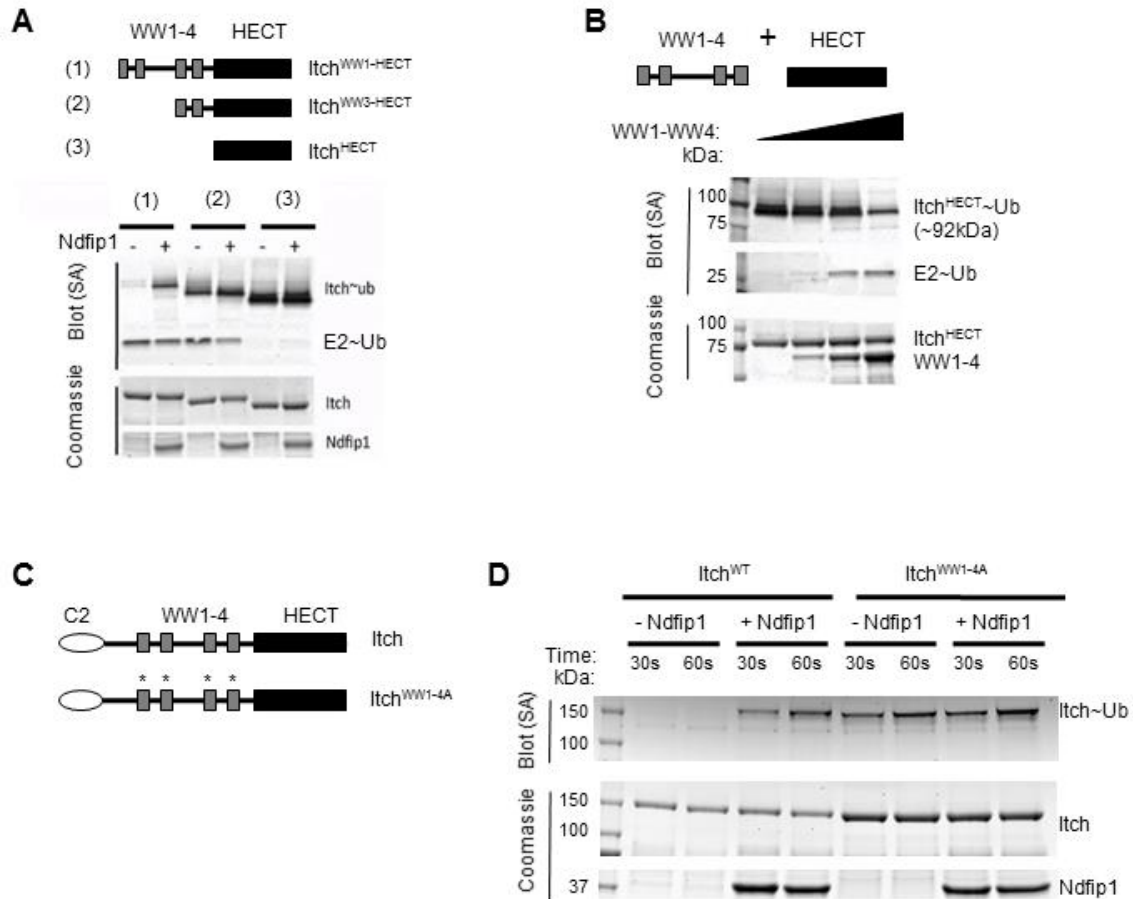


Figure 2.4: Itch autoinhibition is due to an intramolecular interaction between its WW and HECT domains.

A. Itch constructs were generated that lack two or all four WW domains as illustrated. Constructs were assessed for autoinhibition using the ubiquitin charging assay. Streptavidin shows ubiquitylated proteins and coomassie shows loading controls. B. Cassettes containing all four WW domains or the HECT domain were generated as depicted. Ubiquitin charging of the HECT domain (5 μ M) was assessed in the presence of increasing concentrations of the WW domain cassette (0, 2.5, 5, 10 μ M Itch^{WW1-WW4}). Again, streptavidin shows ubiquitylated proteins and coomassie shows loading controls. C. Full length Itch and a construct in which the following point mutations were made in the binding pocket of each WW domain; W315A, W347A, W427A, Y467A (indicated by * and together termed Itch^{WW1-4A}) are illustrated. D. These constructs were analyzed for their ability to accept ubiquitin in the presence or absence of Ndfip1 using the ubiquitin charging assay. For panel A, B and D, similar results were obtained in at least 3 independent experiments.

HECT domain PY motifs are not sufficient for Itch autoinhibition.

It has been proposed that autoinhibition of Nedd4-2 is due to a PY motif in its HECT domain(102,167,173). Notably, this PY motif is buried in the C-lobe conformation observed in all HECT domain structures solved to date, although an isolated peptide binds the NEDD4L WW3

domain as in other LPXY motif-WW domain crystals(102,167). A comparison of the Nedd4-family HECT domains revealed that this PY motif, as well as a second PY motif, is conserved among multiple Nedd4 family members (Figure 2.5A). As PY motifs can bind Nedd4-family WW domains, I asked whether one or both of these HECT domain PY motifs mediated Itch autoinhibition. To determine this, I tested ubiquitin charging of full length Itch with point mutations in these conserved HECT PY motifs (Itch^{Y625A}, Itch^{Y841A}, and Itch^{2PYA}, Figure 5B-D). The amino acid sequence for Itch I refer to can be accessed through the UniProtKB database under UniProtKB accession #Q8C863.2(174). I determined that these mutations in the Itch HECT PY motifs did not relieve autoinhibition, suggesting that either these PY motifs played no role, or that these PY motifs worked together with other motifs.

Figure 5

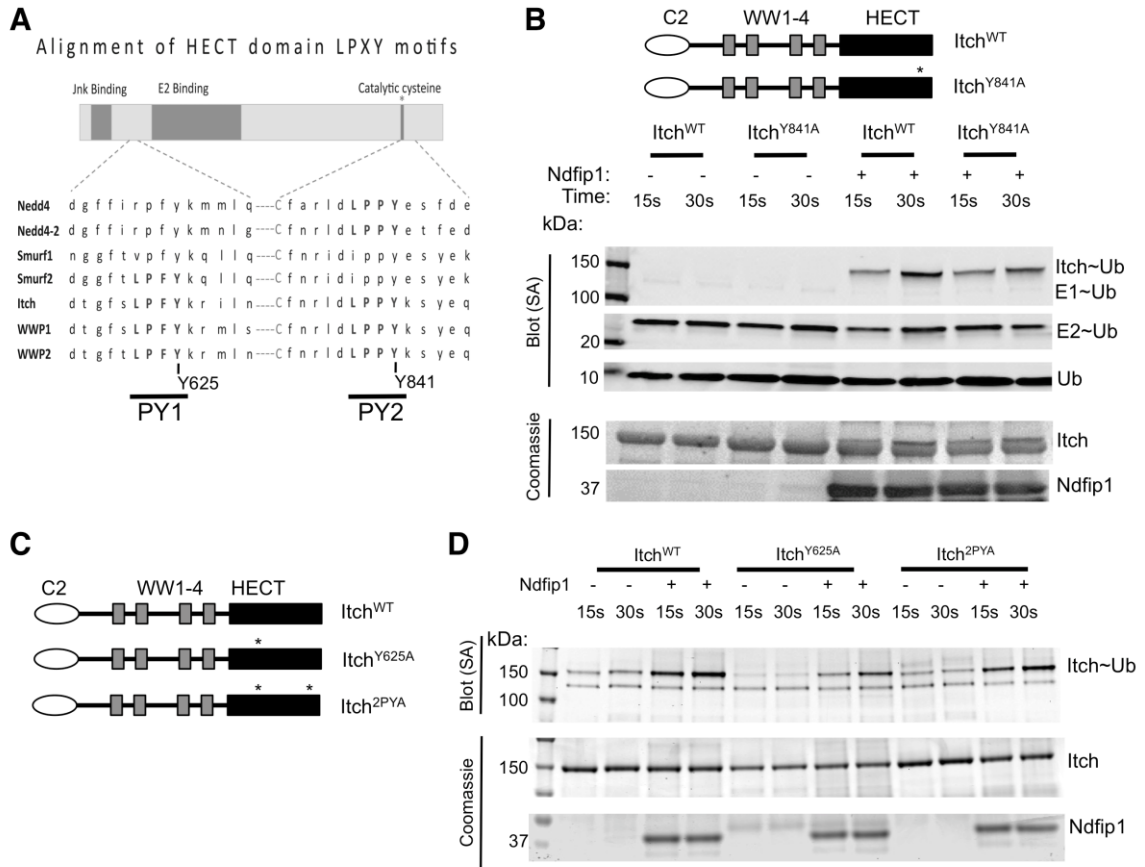


Figure 2.5: The PY motifs in HECT domain are not sufficient for Itch autoinhibition.

A. Alignment of the HECT domains of Nedd4-family members illustrates the conservation of 2 PY motifs. B. A point mutation in Itch, changing the tyrosine in the second HECT PY to alanine, is illustrated. This mutant was tested using the ubiquitin charging reaction in the presence or absence of Ndfip1 and visualized by streptavidin. Loading controls for Itch and Ndfip1 are also shown. C. Tyrosine to alanine point mutations in the HECT PY1 were generated as was a mutant containing both PY Y>A mutations, illustrated in C. D. These were tested as described in panel B. For panel B and D, similar results were obtained in at least 3 independent experiments.

To investigate potential noncanonical or cryptic WW domain binding motifs in the HECT domain, an overlapping peptide array was incubated with MBP tagged WW1-4 region and binding of the WW domains to the peptide array was detected using an antibody recognizing MBP (Figure 2.6A). I identified three regions with the potential to bind Itch WW domains – region 1 was a YFRFIGRF motif (residues 598-605), region 2 was the LPFY motif I had analyzed in Figure 2.5 (residues 622-625), and region 3 was a HYTRTSKQ motif (residues 762-769) (Figure 2.6B,C). As I had previously determined that mutating the tyrosine in region 2 was not sufficient to relieve autoinhibition, I

investigated the contributions of the individual residues in motifs of regions 1 and 3 by alanine scanning (Figure 2.6D). In region 1, mutation of R600 or R604 resulted in diminished binding of the WW domain cassette, while in region 3, mutation of the Y763 residue resulted in decreased WW domain binding (Figure 2.6D,E), demonstrating potential to mediate autoinhibition.

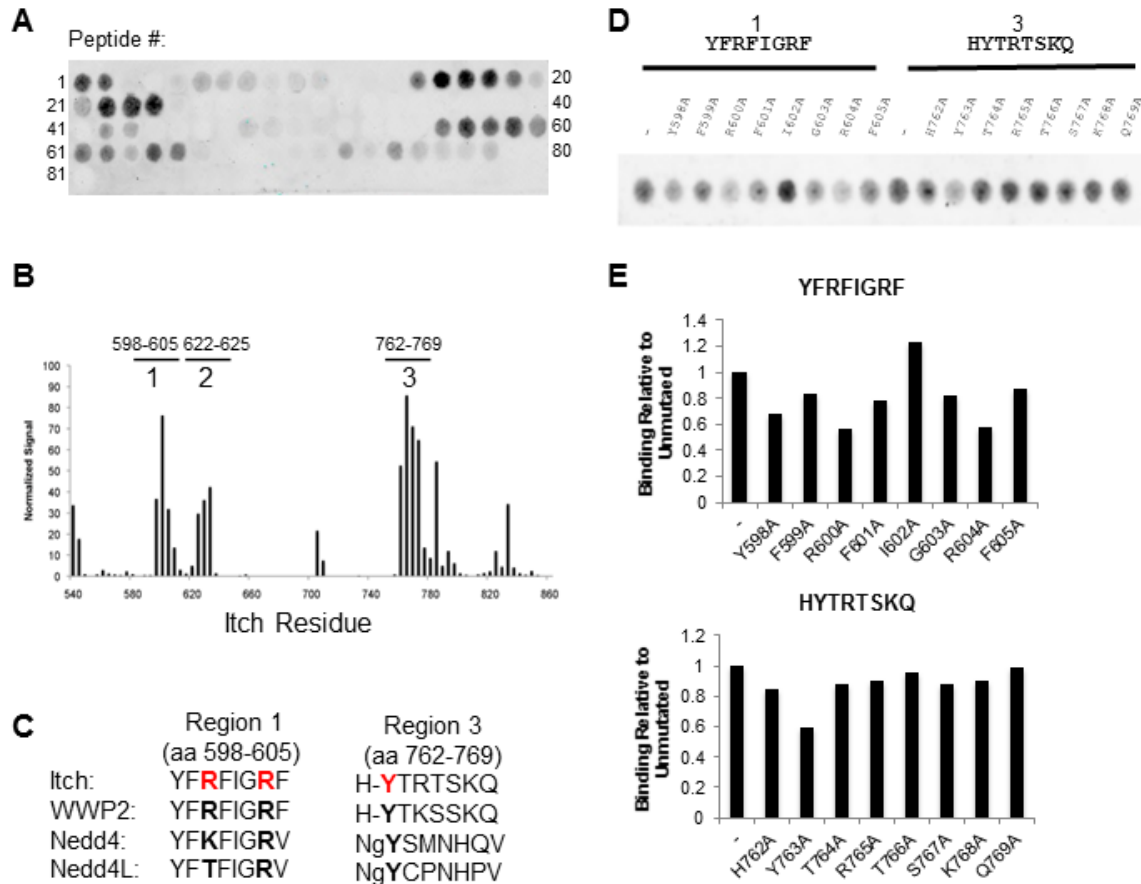


Figure 2.6: WW domains can bind several regions in the HECT domain.

A. An overlapping peptide array spanning the sequence of the Itch HECT domain, consisting of 81 peptides (16 amino acids in length and overlapping by 12 amino acids), was blocked and then incubated with MBP tagged Itch^{WW1-4}. Binding was then analyzed by detection of MBP tag. B. Quantification of A aligned to Itch HECT domain residues. C. Alignment of putative binding regions 1 and 3 between Nedd4 family members. D. Peptide array displaying alanine mutants of binding regions seen in overlapping peptide array were blocked and then incubated with MBP-Itch^{WW1-4}. Binding was then analyzed by detection of MBP. E. Quantification of D.

We attempted to determine contributions of these motifs to autoinhibition by making point mutations in the HECT domain, mutating residue R600 to an alanine in region 1 or residue Y763 to an alanine

in region 3. I then tested the activity of these mutants in a ubiquitin charging assay (Figure 2.7A). Unfortunately, these constructs were not functional, as they were not charged with ubiquitin even upon inclusion of Ndfip1 (Figure 2.7A). I therefore developed a panel of HECT domains in which either single or multiple binding motif residues were mutated to alanine to test how these regions could potentially impact binding. I co-expressed these HECT domain mutants with Itch^{WW1-4}, and assayed co-precipitation of Itch^{WW1-4} with the HECT domain (Figure 2.7B). Using this approach, I determined that mutations of region 2 impaired WW – HECT binding, while mutations in regions 1 or 3 revealed no significant difference in co-precipitation of Itch^{WW1-4}.

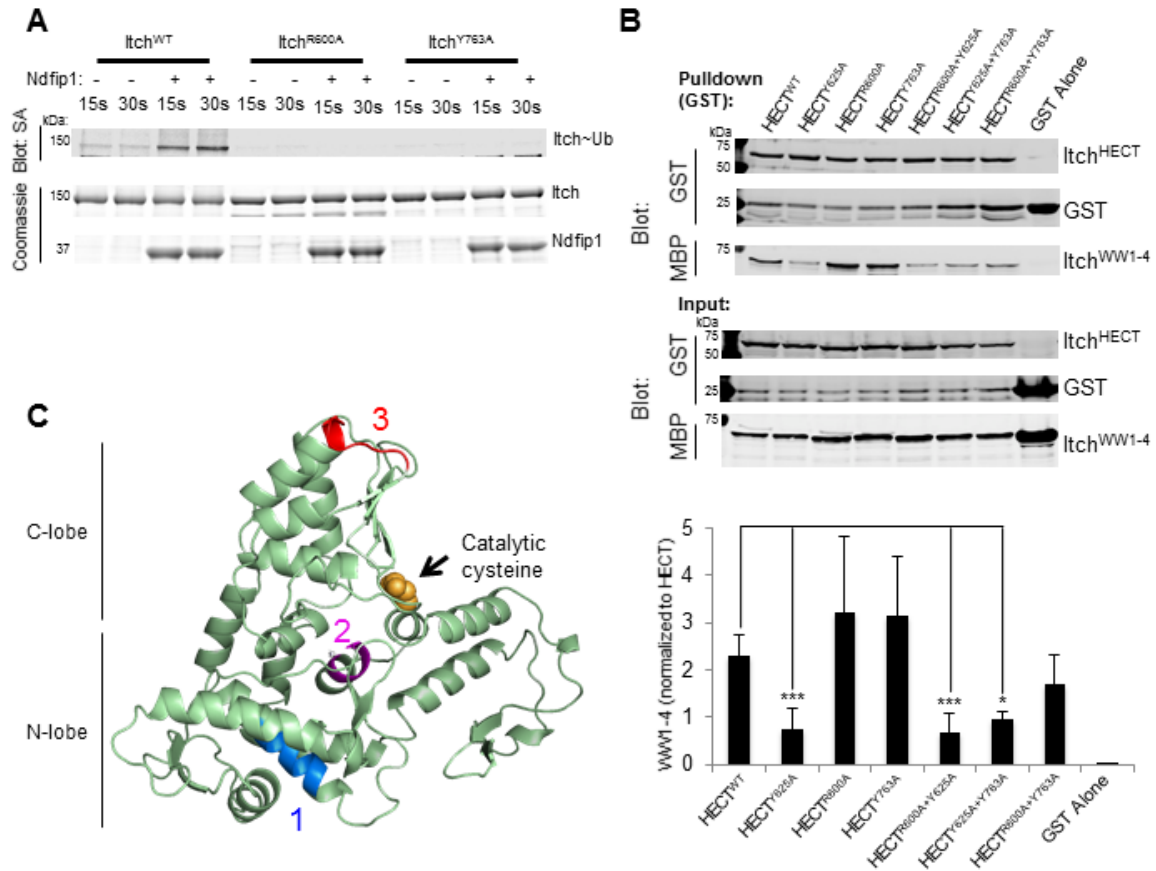


Figure 2.7: Binding analysis reveals a motif in the HECT domain required for WW domain interactions.
A. Ubiquitin charging of Itch^{WT}, Itch^{R600A} and Itch^{Y763A} in the presence or absence of Ndfip1. Itch constructs were incubated in the presence of (biotinylated) ubiquitin-charged E2 for indicated timepoints. Timepoints were analyzed by Western blot using a streptavidin-conjugated fluorophore highlighting ubiquitin-charged Itch species. B. Top: MBP-tagged Itch^{WW1-4} was co-expressed with a panel of GST-tagged Itch^{HECT} mutants and co-precipitation of MBP-Itch^{WW1-4} with each HECT was analyzed. Bottom: Quantification of blots. Itch^{WW1-4} co-precipitation was normalized to Itch^{HECT} (mean \pm SEM, two-tail T-test). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. C. Itch HECT domain structure highlighting region 1 in blue, 2 in pink, and 3 in red. For panels A and B, similar results were obtained in at least 3 independent experiments.

As with the previously identified region of NEDD4L that binds the WW3 domain, all the sequences I identified are embedded in folded structures within the ITCH HECT domain. Notably, computational studies of NEDD4L revealed the potential for local unfolding of the HECT domain to reveal cryptic linear motifs that could mediate autoinhibition(102). Although it is impossible to model these regions in the context of interaction with a WW domain, I have highlighted their relative locations in Figure 2.7C. Overall, our data raise the possibility that region 2 could play an important

role mediating HECT – WW binding. Future structural studies are needed to determine how this region or other as yet unidentified regions in the HECT domain could mediate autoinhibition.

Ndfip1 binds Itch WW domains to mediate autoinhibition.

To determine how Ndfip1 relieves autoinhibition, I asked whether the addition of Ndfip1 could prevent WW domain-mediated inhibition of the Itch^{HECT} domain 'in-trans'. I repeated the experiment in Figure 2.4B, but this time in the presence of Ndfip1 (Figure 2.8A). Ndfip1 prevented WW domain-mediated inhibition of the Itch^{HECT} domain. Furthermore, the WW domain cassette lacking functional WW domains was unable to support autoinhibition, and addition of Ndfip1 to this reaction had no effect. This fits with published data showing that Ndfip1 binds the WW domains of Itch(105). Nevertheless, to show that Ndfip1 binds exclusively to the WW domains, and not the HECT, I co-expressed various combinations of these cassettes and used a pull-down approach to test whether Ndfip1 could also bind the HECT domain. Consistent with previous studies, I found that Ndfip1 associated with WW1-4, but not the Itch^{HECT} domain (Figure 2.8B).

To determine which WW domains were preferred for Ndfip1 interactions, I tested several mutants of Itch that contained point mutations in one or two WW domains. I identified one mutant that showed a significant decrease in Ndfip1 binding. Specifically, mutating WW domains 2 and 3 (Itch^{WW2,3A}) was sufficient to interrupt most of the interaction between Itch and Ndfip1 (Figure 2.8C). To determine whether this minimal mutation was also sufficient to relieve autoinhibition of Itch, I used an autoubiquitylation assay that measures ubiquitin chain formation by Itch. This approach can analyze the formation of ubiquitin chains over time, by measuring the fluorescence resonance energy transfer (FRET) that occurs when ubiquitin chains are generated. This high-throughput assay also offers the advantage of monitoring the time-courses of ubiquitin chain formation with many variables assayed simultaneously. Using this assay, I determined that Itch^{WW2,3A} was active in the absence of Ndfip1 (Figure 2.8D). This was in stark contrast to Itch^{WT}, which was inactive for the duration of the assay, a finding consistent with our ubiquitin charging results showing no transthiolation in the absence of Ndfip1. Importantly, Itch^{WW2,3A} was as active as Itch^{WW1-4A}, the

mutant determined previously to be relieved of autoinhibition. These data indicate that Ndfip1 activates Itch by relieving an intramolecular interaction between the Itch WW and HECT domains that restricts its catalytic function.

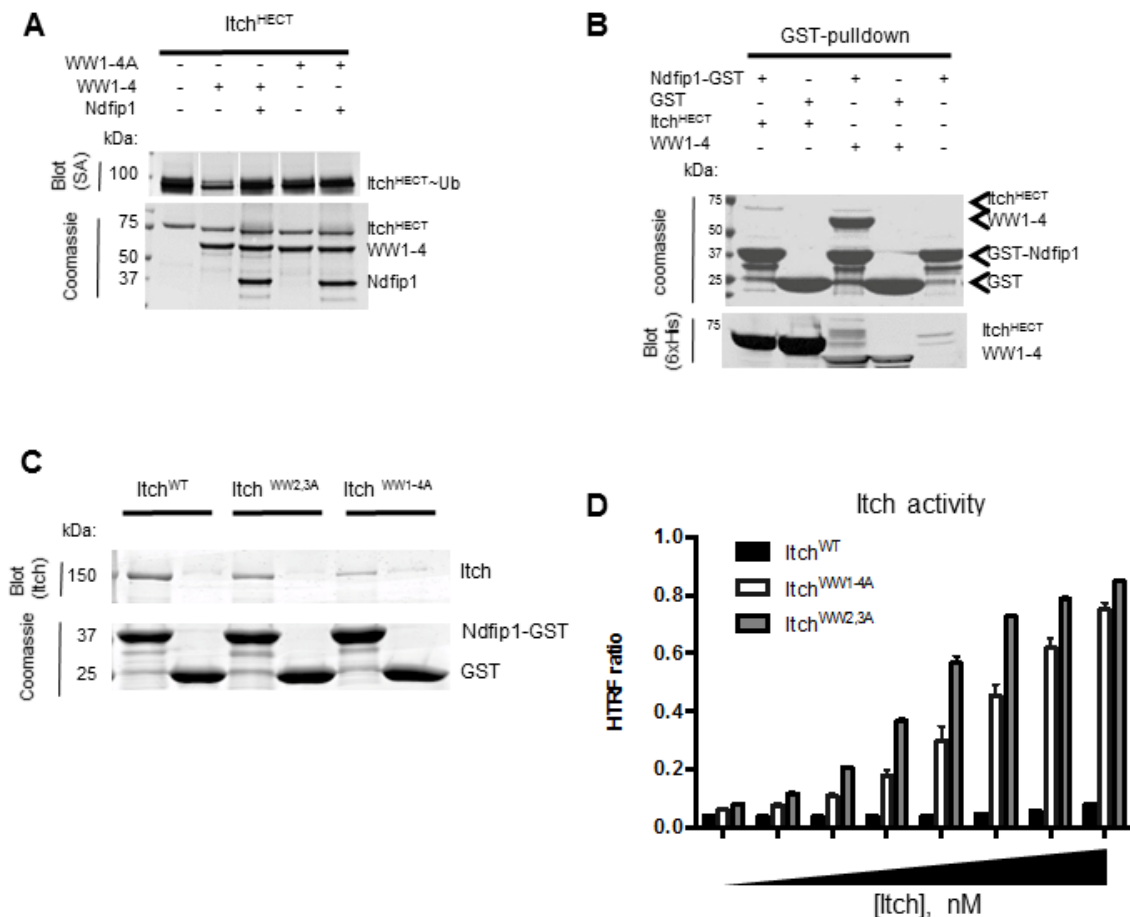


Figure 2.8: Ndfip1 relieves autoinhibition by binding Itch WW domains.

A. Ubiquitin charging of the HECT domain was tested alone (lane 1) or in the presence of WT WW domains (WW1-4, lanes 2 and 3) or mutant WW domains (WW1-4A, lanes 4 and 5) and in the presence or absence of Ndfip1 (lanes 3 and 5). Detection of the ubiquitin charged HECT domain is by streptavidin. B. GST-tagged Ndfip1 or GST alone was pulled down from cells also expressing the Itch His-tagged HECT or WW1-4 domains. Enriched proteins were visualized by coomassie and domain expression was confirmed in bacterial lysates using anti-His. Cells expressing GST-Ndfip1, but no Itch domain, provide a negative control. C. GST-tagged Ndfip1 or GST alone was pulled down from bacteria expressing Itch, Itch^{WW2,3A}, or Itch^{WW1-4A}. Proteins were visualized by coomassie and Itch construct expression was confirmed using anti-Itch. D. Activity of Itch, Itch^{WW2,3A}, and Itch^{WW1-4A} was assayed using a homogeneous FRET assay. For all panels, similar results were obtained in at least 3 independent experiments.

Mutating WW domains 2 and 3 results in increased Itch function in cells.

We next asked whether relief of autoinhibition was sufficient to increase Itch activity in cells. Itch has been shown to promote ubiquitin-mediated degradation of JunB, an AP-1 transcription factor component that promotes IL-2 and IL-4 production(93,97,104,105). In order to examine the effects of Itch^{WW2,3A} on JunB stability, I expressed Itch^{WT} or Itch^{WW2,3A} in Jurkat cells, an immortalized T

lymphocyte line, and assessed JunB stability after stimulation (Figure 2.9A). Both Itch^{WT} and Itch^{WW2,3A} showed high levels of expression. However, Itch^{WW2,3A} levels were consistently lower than levels of the Itch^{WT} counterpart. Cycloheximide treatment indicated that this was due to increased degradation of Itch^{WW2,3A} (Figure 2.9B). Despite the reduced level of Itch^{WW2,3A}, I observed that JunB degradation was significantly increased in cells expressing Itch^{WW2,3A} compared to those expressing Itch^{WT} (Figure 2.9C). Supporting this, expression of these mutants in HEK293T cells also showed that constitutively activate Itch is less stable, whereas the catalytically inactive version of Itch^{WW2,3A} was stabilized (Figure 2.9D,E). This highlights that Itch^{WW2,3A} instability is mediated by its catalytic activity, as high Itch^{WW2,3A} activity resulted in its instability. Furthermore, this indicates that while Ndfip1 might act to recruit an E2(175) under some circumstances, the primary function of Ndfip1 is to relieve Itch autoinhibition.

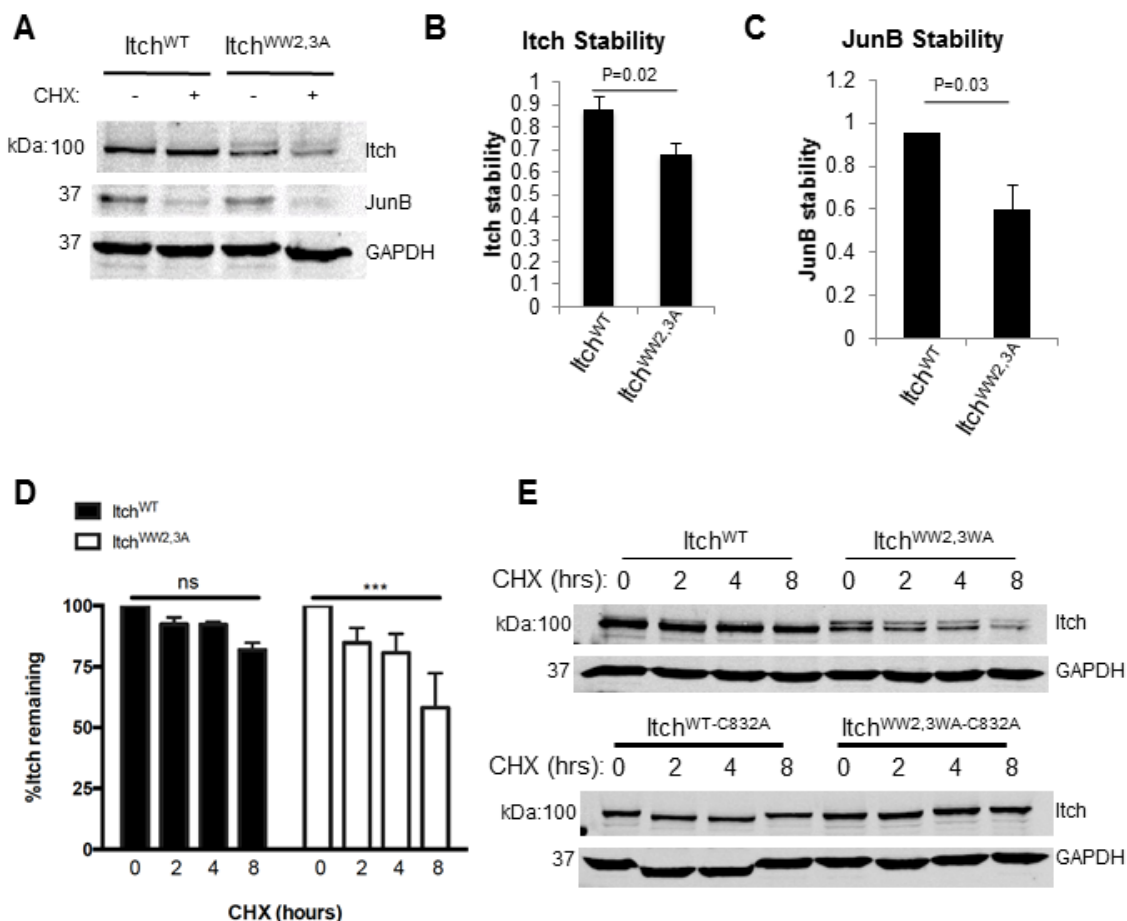


Figure 2.9: Relieving autoinhibition of Itch is sufficient to facilitate substrate degradation.

A. Jurkat cells transfected with Itch^{WT} or Itch^{WW2,3A} were stimulated and then treated with cycloheximide. Cells were harvested and expression of Itch and JunB was analyzed by Western blot. B. Itch stability over 4 hour cycloheximide treatment, normalized to GAPDH expression (mean ± SD, two-tail T-test). C. Degradation of JunB relative to expression of Itch or Itch^{WW2,3A} mutant. JunB levels were normalized to GAPDH. Loss of JunB following cycloheximide treatment is shown as a fold change of Itch^{WW2,3A} relative to cells expressing Itch^{WT} (mean ± SEM, single sample T-test). D and E. Itch stability was analyzed in HEK293T cells as in panel A but with the addition of constructs in which Itch catalytic activity is ablated (C832A mutation). Percent Itch remaining was quantified for Itch^{WT} and Itch^{WW2,3A} at indicated time point (mean ± SEM, P ≤ 0.001 by 2 way ANOVA). For all panels, similar results were obtained in at least 3 independent experiments.

Ndfip1 is required for some but not all Nedd4-family members.

To identify the minimal motifs in Ndfip1 needed to activate Itch, peptide mimics of Ndfip1 were tested. I generated synthetic peptides that contain 1, 2, or all 3 PY motifs of Ndfip1 (Figure 2.10A). There is a striking increase in Itch activity as the number of PY motifs is increased (Figure 2.10B). While these data show that Ndfip1 promotes Itch activation, all Nedd4 family members contain

between 2 and 4 WW domains, thus the intramolecular interaction mediated by WW domains could be a common means of Nedd4-family autoinhibition(90). To test whether Ndfip1 could promote the activity of other Nedd4-family members I again employed the FRET-based assay to monitor ubiquitin chain formation. Addition of Ndfip1 substantially activated both ITCH and WWP2 in a dose dependent manner. Additionally, I tested the prototypic family member Nedd4. Interestingly, Nedd4 was active in these assays without the addition of Ndfip1. The addition of Ndfip1 modestly, but consistently, potentiated the formation of ubiquitin chains (Figure 2.10C), but these differences were only observed at early time points. These differences could be explained by the increased activity of E3s when associated with potential substrates as described in *Kamadurai et al.(176)* Based on these data, I propose a model of how Itch and a subset of Nedd4-family members are autoinhibited and can be activated by Ndfip1 (Figure 3.1). This model advances our understanding of how Itch and related Nedd4-family members are held in an 'off' position, and how they are activated, by defining the structural features of autoinhibition and Ndfip1 activation.

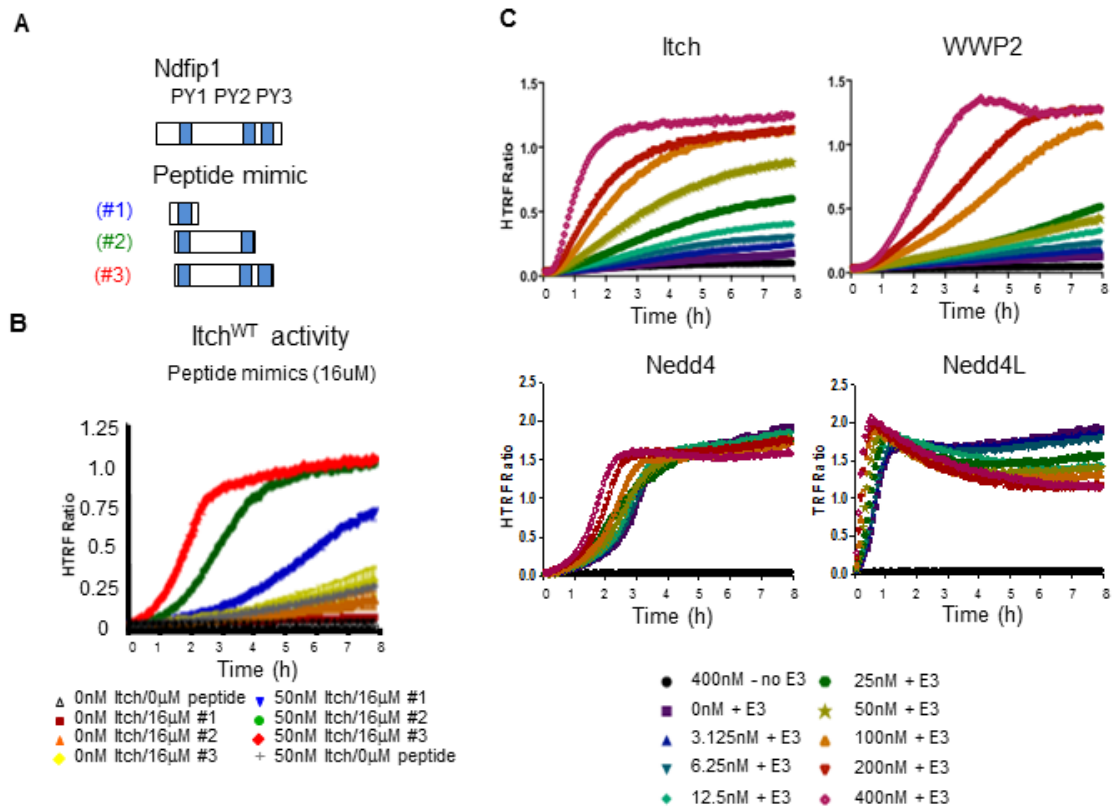


Figure 2.10: Ndfip1 and its peptide mimics are required to activate a distinct subset of Nedd4-family members.

A. Cartoon representation of peptides generated that span one, two or all three PY motifs of Ndfip1. B. Peptides shown in panel A were tested for potential to promote Itch polyubiquitylation activity in a time-resolved FRET assay (TR-FRET). Briefly, an *in vitro* ubiquitylation assay was performed in a 392-well plate using fluorescein-conjugated ubiquitin and a terbium-conjugated tandem ubiquitin binding entity (TUBE). The TUBE binds polyubiquitin chains preferentially, and FRET activity of the interacting terbium-fluorescein pair is measured as a readout of ubiquitylation activity. Multiple plate reads were analyzed with each plate read occurring at 4-minute intervals. Data is representative of a minimum of 3 individual experiments. C. Ndfip1 was analyzed for its ability to activate Itch, WWP2, Nedd4 and Nedd4L using the TR-FRET assay. For panels B and C, similar results were obtained in at least 3 independent experiments.

Discussion

Proper orchestration of the ubiquitylation cascade is critical for cellular functions. Catalytic E3 ubiquitin ligases, which provide substrate specificity and can also catalyze the addition of ubiquitin to target proteins, are highly regulated through various autoinhibitory mechanisms. RING E3s can

be inhibited through intramolecular associations that obstruct functions required for the ligase activity such as substrate binding in UBR1(177), dimerization in cIAP1(178), and E2 binding in Cbl-b(179). Even the multi-subunit RING E3 SCF can be inhibited by CUL1's extreme C-terminus interacting with the Rbx1 RING domain(180). Thioester-forming E3 ligases such as RING-between-RINGs (RBRs) and bacterial HECT-like E3s are subject to autoinhibition through multiple means, including E2 recruitment/binding, and impairment of E3~Ub thioester intermediate formation. In RBRs, distinct regions mask the active-site cysteine requiring structural rearrangements for activity(181-184), whereas in bacterial SspH1, the inhibitory leucine-rich repeat (LRR) domain inhibits ubiquitin transfer to targets after E3 transthiolation(185). Of the thioester-forming Nedd4-family members, Smurf2 has the most defined mechanisms of autoinhibition. Smurf2 function is tightly orchestrated by its adaptor Smad7(186), as Smurf2 activity is restrained by both an intramolecular interaction and by a suboptimal E2-binding site within the HECT domain. Smad7 activates Smurf2 in two ways, by bringing it together with its E2(161), and by directing it to the plasma membrane where the C2 releases the HECT domain. It was recently shown that the C2 domain limits Smurf2 function by maintaining the HECT domain in a low activity state in which it does not form a thioester bond with ubiquitin, preventing downstream conjugation of ubiquitin to substrate¹⁴. While crystal structures have provided insight into potential intramolecular interactions, the intrinsically unstructured nature of regions within Nedd4 family members has prevented a complete picture of autoinhibited structures as only individual, stable domains can be crystallized.

Activation of Nedd4-family members can be accomplished through changes in the intracellular milieu, phosphorylation, and adaptor protein interactions. Intramolecular inhibitory interactions between the C2 and HECT domains of NEDD4 are relieved by phosphorylation of two tyrosine residues, one found in the C2 domain and another in the HECT domain, activating NEDD4 catalytic activity(167). Nedd4 autoinhibition is relieved by C2 domain mediated calcium binding, which may also promote membrane association(100), while Nedd4-2 phosphorylation by serum and glucocorticoid-regulated kinase promotes SGK ubiquitylation and subsequent degradation(166).

Smurf2, as described previously, is regulated both by Smad7 binding(99,161) and an intramolecular interaction between the C2 and HECT domains.

Itch can be activated by both phosphorylation and by association with adaptor proteins(93,104,105). Itch is activated by JNK1-mediated phosphorylation, an event that has been mapped to a unique region in Itch between its C2 and first WW domain(104). Itch is also regulated by interactions with adaptor proteins, such as Nedd4-family interacting proteins 1 and 2. Therefore, I took a reductive approach to isolate the mechanism by which Ndfip1 binding alleviates Itch autoinhibition.

Here, I provide new information on how autoinhibition prevents the activation of several related Nedd4 family E3 ubiquitin ligases and show how Ndfip1 activates Itch, two processes that are functionally linked. Our data reveal the regions in Itch that mediate autoinhibition, namely the WW domains 2 and 3, and that autoinhibition is achieved via blocking E2-E3 transthiolation. These two WW domains bind the Itch HECT domain, preventing the transfer of ubiquitin to the catalytic cysteine of Itch. This autoinhibition holds Itch in an 'off' position and maintains Itch stability. Supporting this, I have shown that Itch activation is tightly linked to its stability (Figure 9). Thus, regulation of cellular Itch levels depends on adapter proteins such as Ndfip1 and Ndfip2.

While it is clear that two Itch WW domains bind the HECT domain, the precise regions in the HECT that are used for binding remain unclear. Related challenges have been encountered in attempts to understand mechanisms underlying autoinhibition of other Nedd4-family HECT E3s, although recent breakthrough studies revealed the potential for local flexibility to expose otherwise folded linear motifs within HECT domains(102,167,173). I have determined one region, a PY motif (amino acids 622-625), which supports binding between the HECT and WW domains. However, mutation of this region was not sufficient to relieve autoinhibition. Considering that two WW domains are needed to maintain autoinhibition, it is likely that the WW domains bind the PY motif as well as another region. Using a peptide array, I identified two other motifs in the HECT that were able bind the Itch WW domains, however subsequent mutations of these regions did not reduce HECT-WW

binding. Thus, in the future, a more detailed structural analysis will be needed to precisely define HECT – WW binding.

I show that Ndfip1 binding to Itch relieves autoinhibition, allowing the transthiolation reaction between E2 and E3 to occur. Additionally, I show that multiple PY motifs are required for Ndfip1 to activate Itch. I propose that PY binding motif number could functionally distinguish a protein that is an activator of Itch from one that is a substrate. Supporting this, I have tested Ndfip2 and have determined that it, like Ndfip1, promotes ubiquitin charging of Itch (Figure 2.1, C). Interestingly, there is evidence that viral proteins may coopt this mechanism, as the Epstein-Barr virus LMP2A requires two PY motifs in order to activate the human Itch homologue, AIP4(187). My data clarifies that, in the autoinhibited state, Itch can bind to an E2, but it is not until interaction with an activator like Ndfip1 that it is capable of ubiquitylation. My model also illustrates that Ndfip1, via multiple PY motifs, binds to the same WW domains that are necessary for autoinhibition. This may suggest a paradigm for other E3/adaptor interactions. Additionally, these mechanistic insights also apply to other Nedd4 family members, namely WWP2.

Although at this point it is not possible to make a detailed structural model, this work builds on emerging principles based on structural and other studies of HECT E3s. This work reveals new intramolecular interactions that target the HECT domain to mediate autoinhibition. Previous work has shown that Nedd4, Nedd4L (Nedd4-2), and Smurf2 mediate autoinhibition through a C2 domain – HECT domain interaction(99,101,102,167). I show that Itch autoinhibition is mediated by WW - HECT domain interactions. Identification of such distinct mechanisms reveals how cells have evolved unique ways to activate different Nedd-4 family members, leading to differential function of proteins with high structural similarity. Furthermore, these distinct inhibitory mechanisms could be used to develop therapeutics that activate some, but not all, Nedd4-family E3 ubiquitin ligases, allowing specificity and thus resulting in fewer off-target effects.

Methods

Protein expression and purification - Nedd4 family member E3 ligase constructs were prepared using PCR amplification to insert restriction sites in cDNA encoding specified variants. cDNA constructs used: mouse Itch variant 1 (MC205232, Origene), mouse Ndfip1 (MG202546, Origene), mouse Nedd4 (MG222243, Origene), mouse WWP2 (MC202685, Origene). These inserts were ligated into an N-terminal His-MBP tag pRSF-1B plasmid. WW domain mutants were made by introducing a tryptophan to alanine point mutation in the hydrophobic binding pocket. Truncation mutants of Itch were constructed from mouse Itch cDNA. ItchWW1-HECT construct contains residues 285-864. ItchWW3-HECT contains residues 397-864, and ItchHECT contains residues 486-864. Site directed mutagenesis was performed using the Quikchange Mutagenesis Kit (Agilent). Ubch5B and Ubch7 fused with SUMO and a hexahistidine tag were cloned into pRSF-duet expression vectors. GST-tagged full length Itch and HECT domain were cloned into pGEX 4T-1 to provide GST-Itch for affinity studies. Ndfip family constructs were prepared as previously described and ligated into pGEX-4T-2 vector (GE Life Sciences). Plasmids were transformed into BL21(DE3) E. coli and purified with Ni-NTA agarose (Qiagen) in the case of His-MBP fusion proteins or glutathione sepharose 4B (GE Life Sciences) in the case of GST fusion proteins followed by size-exclusion chromatography.

Octet BioLayer Interferometry (BLI) and binding kinetics - Anti-GST antibody-conjugated biosensors were used to trap GST-ITCH as ligand. 1-50 μ M of Ubch5B and 3-250 μ M of Ubch7 as analytes were prepared in assay buffer (25 mM Tris pH 7.6, 150 mM NaCl, 0.1 mg/ml bovine serum albumin and 0.01% tween-20) to titrate the GST-ITCH bound biosensor for measuring the binding kinetics. All experiments were carried out on an Octet Red96 (Pall ForteBio Corp, Menlo Park, CA) with 4 repeats at 26°C. Both association and dissociation steps in the BLI assays were 60 seconds. The response shifts were extracted and fitted to an equivalent binding model to obtain the K_d values.

Ubiquitin Charging Assays - E1 ubiquitin activating enzyme Ube1 (Boston Biochem), E2 ubiquitin conjugating enzyme Ubch7 (Boston Biochem) and biotinylated ubiquitin (Boston Biochem) were incubated at 25°C in ubiquitylation buffer (40 mM Tris 7.6, 250 mM NaCl, 1.7 mM ATP, 8.3 mM MgCl₂, 3.3 µg/ml ovalbumin). After 30 minutes, the reaction was quenched on ice by five-fold dilution with quenching solution (25 mM EDTA, 25 mM HEPES pH 7.5, 100 mM NaCl). The resultant ubiquitin-loaded E2 mix was then introduced to the E3 mix on ice, resulting in a final mix concentration of 6 µM E3, 200 nM E2, 10nM E1, and 400 nM Ub. Ndfip1 constructs introduced to the mix were at a final concentration of 16 µM. WW domain trans inhibition assays used a final concentration of 30 µM WW domain and 60 µM Ndfip1. Loading of ubiquitin onto the E3 via thioester bond formation was stopped by quenching with an equal volume of Laemmli buffer at indicated time points. Ubiquitylated products were resolved on SDS gels and detected using Li-Cor IRDye 800CW Streptavidin (Li-Cor). Confirmation of thioester loading was obtained by introducing samples to 50 µM DTT to reduce the thioester bond.

Homogeneous E3 ubiquitylation assay - E3 ubiquitylation activity was monitored using a homogeneous TR-FRET assay as described previously with slight modifications(27). The reaction components were diluted in assay buffer (50 mM Tris pH 8.0, 5 mM MgCl₂, 1mM β-mercaptoethanol and 0.05% CHAPS) to a final volume of 20 µL and assembled in a 384-well polypropylene plate. Briefly, Ubiquitylation Mix (0.2 mM ATP, 5 nM E1, 100 nM Ubch5c) containing varying concentrations of E3 was combined with varying doses of Ndfip1/2 or peptides and HTRF Detection Mix (Biotin-K63TUBE (LifeSensors), SA-Tb, (CisBio), wild type and FAM-labeled Ubiquitin). TR-FRET was monitored in real time using a PerkinElmer Envision plate reader (Ex 340nm; Em1 520nm; Em2 480nm). The TR-FRET ratio was calculated by Em520/Em480.

Jurkat transfection - ItchWT and the ItchWW2,3A mutant were cloned into the MSCV-IRES-NGFR vector (provided by Dr. Andrew Wells, Children's Hospital of Philadelphia). Cells were transfected using the Invitrogen Neon Electroporation system (Invitrogen) according to manufacturer's protocols. Prior to transfection, Jurkat E6.1 cells (Progenra) were grown in RPMI1640 (Gibco)

supplemented with 10% FCS, 20mM GlutaMAX (Gibco) and 25 mM HEPES pH 7.0 with no antibiotics. Cells were washed with PBS and resuspended at 2.0×10^7 cells/ml in Buffer R (Invitrogen) prior to electroporation. 24 hours later transfected cells were purified based on NGFR expression using PE-NGFR antibody (BD), anti-PE magnetic beads, and LS columns (Miltenyi). To assess protein stability, NGFR+ cells were stimulated for 2 hours with PMA (30ng/ml) and ionomycin (1 μ g/ml), and cycloheximide (Sigma) was added to a final concentration of 10 μ g/ml for an additional two hours of stimulation. JunB was quantified using rabbit anti-JunB (Santa Cruz, SC-44). Degradation of JunB in ItchWW2,3A was then compared to degradation of JunB in ItchWT to normalize for variations in JunB expression.

HEK293T Transfection - ItchWT, ItchC832A, ItchWW2,3A and ItchWW2,3A-C832A in MSCV-IRES-NGFR vector was incubated with Lipofectamine 2000 (Invitrogen). 2 μ g plasmid was incubated with 20 μ l Lipofectamine 2000 for 20 minutes in 1 ml Opti-MEM (Gibco) and then introduced to 70% confluent HEK293T cells on 60mm cell culture dishes (Corning). Cells were then incubated with Lipofectamine and plasmid mixture at 37°C in 5% CO₂ for 4 hours. Transfection media was replaced with growth media and cells were incubated 37°C overnight. Cycloheximide was introduced at a final concentration of 10 μ g/ml and timepoints were harvested every two hours. Cells were washed in PBS and lysed in 50 mM Tris-HCl pH 7.6, 1% NP-40, 150 mM NaCl with Complete Protease inhibitors (Roche). Lysates were run on Biorad Criterion 4-20% Tris-HCl gradient gels at 20 μ g lysate per lane.

Overlapping Peptide Array - PepSpot peptide arrays (JPT peptides) containing 81 overlapping peptides covering the mouse HECT domain (aa530-864) were synthesized. Each peptide was 16 residues in length and overlapped adjacent sequences by 12 residues. The PepSpot array was activated in methanol, washed in TBS and then blocked for 3 hours in Western Blocking Reagent solution (Roche). MBP-tagged WW1-WW4 cassette was then added at 10 μ g/ml and incubated at 4°C overnight. MBP was detected using a mouse monoclonal anti-MBP antibody (NEB) and IRDye

800 CW anti-mouse IgG (Li-Cor). Binding was quantified using laser densitometry on the Li-Cor Odyssey system.

Peptide mimic synthesis - Ndfip1 peptide mimics were synthesized by the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT, USA). Peptides were purified using reverse phase HPLC and mass confirmed by MALDI-TOF and LC-MS. Lyophilized peptides were resuspended in water, incubated at 37°C for 1 hour and vortexed. Peptide amino acid sequences were as follows: Peptide #1: N-PEQTAGDAPPPYSSITAESAAYFDY-C. Peptide #2: N-GDAPPPYSSITAESAAYFDYKDESGFPKPPSYNVA-C. Peptide #3: N-GDAPPPYSSITAESAAYFDYKDESGFPKPPSYNVATTLPYDEAE.-C

Chapter 3 - Discussion

Nedd4 family member interactions are directed both by attributes intrinsic to the domains of which they are comprised and the intramolecular interactions these domains engage in. While our understanding of how these domains act in isolation has increased, our understanding of the intramolecular interactions these domains participate in, and how they determine the overarching characteristics of the Nedd4 family member in question, is limited. Here I present findings that illustrate a novel intramolecular interaction regulating Itch and related Nedd4 family member activation that distinguish these E3s as functionally distinct from other Nedd4 family members. While the C2 domain regulates autoinhibition of Nedd4 and Nedd4L, and contributes to autoinhibition of Smurfs 1 & 2, my work has determined that Itch WW domains regulate its autoinhibition. I have shown that Itch autoinhibition is achieved by preventing a transthiolation event, blocking the transfer of ubiquitin from an E2 to the HECT domain, and that an E2 was able to bind to autoinhibited Itch in that absence of Ndfips. I found that this autoinhibition requires interaction with an adaptor containing multiple PY motifs in order to be relieved, and that single PY motifs in isolation are not capable of activating Itch (Figure 3.1). By mutating the regions of the WW domains that mediate autoinhibition, I have shown that constitutively active Itch exhibits a marked reduction in stability. I found that this reduction in stability is mediated by the catalytic activity of Itch itself. Thus, catalytic activity of Itch is restrained, and could therefore be regulated, by interaction of WW domains with the HECT domain. Additionally, I show that similar mechanisms are likely to regulate activation of WWP2, and, by inference, the closely related WWP1. The implications of this WW domain mediated mechanism of autoinhibition, its interaction with E2s, and the role of Ndfips in Itch activation are discussed in this chapter.

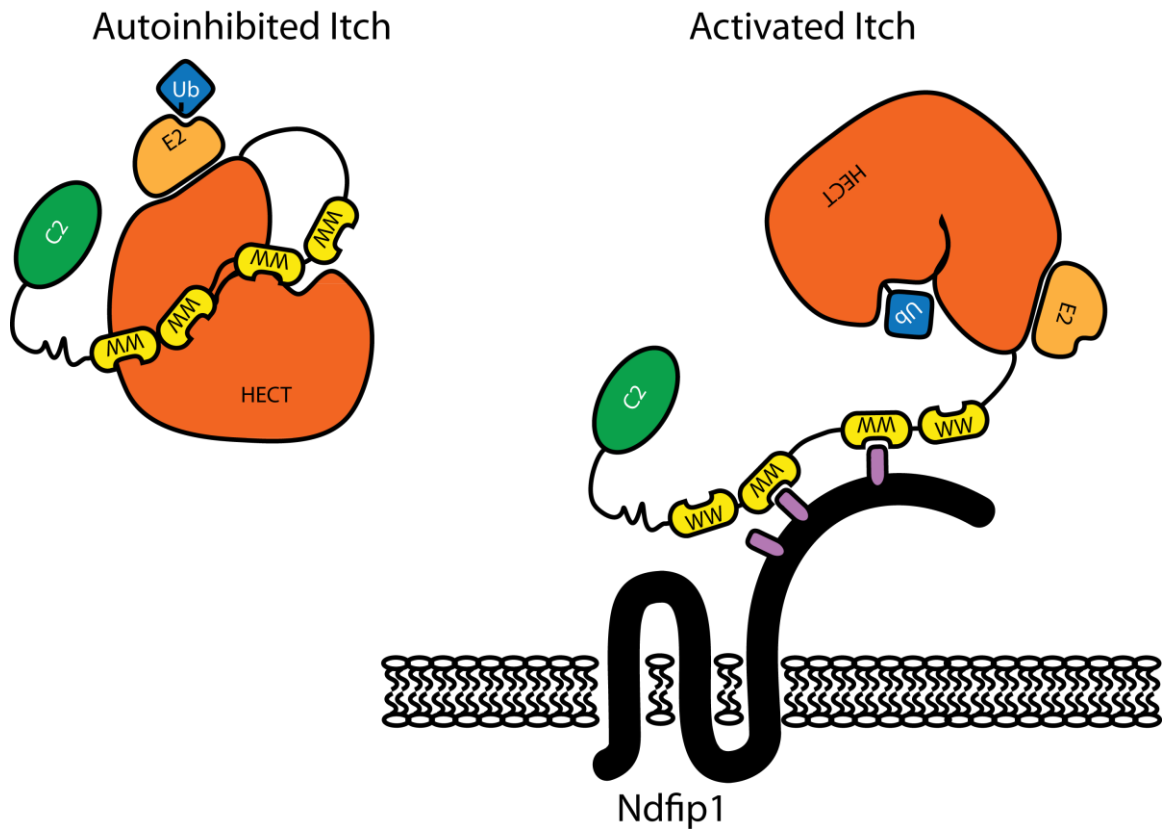


Figure 3.1: A model of Itch autoinhibition and Ndfip1-mediated activation of Itch.

Itch, though free to interact with ubiquitin charged E2, is autoinhibited by an intramolecular interaction between its HECT domain and its WW domains. Ndfip1 binds multiple WW domains via its PY motifs, relieving autoinhibition and facilitating transthiolation and ubiquitin charging.

A model of Nedd4 subfamily division.

The general feature of an intramolecular autoinhibition mechanism is well preserved among Nedd4 family members. Broadly, in terms of the HECT domain activation, we can categorize Nedd4 subfamilies as C2 inhibited (Nedd4 and Nedd4L), E2 inhibited (Smurfs), or WW domain inhibited (Itch, WWP1 and WWP2) (Figure 3.2). Smurf2, Nedd4L, and Nedd4 regulate HECT domain activity in a similar manner. The C2 domain interacts with a binding face on the N-lobe of the HECT domain, blocking formation of a thioester-bonded ubiquitin to the catalytic cysteine(101,102,167). The tyrosine residue that mediates HECT-C2 domain interactions in Nedd4 and Smurf2 (Y604 and Y453, respectively) is absent in the Itch-WWP subfamily, instead substituted by a methionine. Additionally the I36/L37 residues of the Nedd4 C2 domain that mediate this interaction, while

conserved in Nedd4, Nedd4L, and Smurfs 1 & 2, are replaced by polar residues in the corresponding position in the Itch-WWP subfamily. C2-HECT mediated intramolecular autoinhibition can be alleviated in a variety of ways that illustrate the underlying functional division of Nedd4 subfamilies. Calcium binding to the C2 domain of Nedd4 relieves autoinhibition, as does phosphorylation of either the interacting face of the HECT domain or the C2 domain(167). Smurf2, while inhibited through the C2 domain, is activated through a two-stage process in which an open conformation is achieved through a PY motif-WW domain interaction and an E2 is recruited to the HECT domain(99,161). My work has shown that the Itch-WWP subfamily autoinhibition is primarily mediated by WW domain interactions. This has certain implications regarding the Itch-WWP subfamily, as it supports that Itch-WWPs are not as reliant on calcium mobilization as the C2-domain regulated Nedd4 family members.

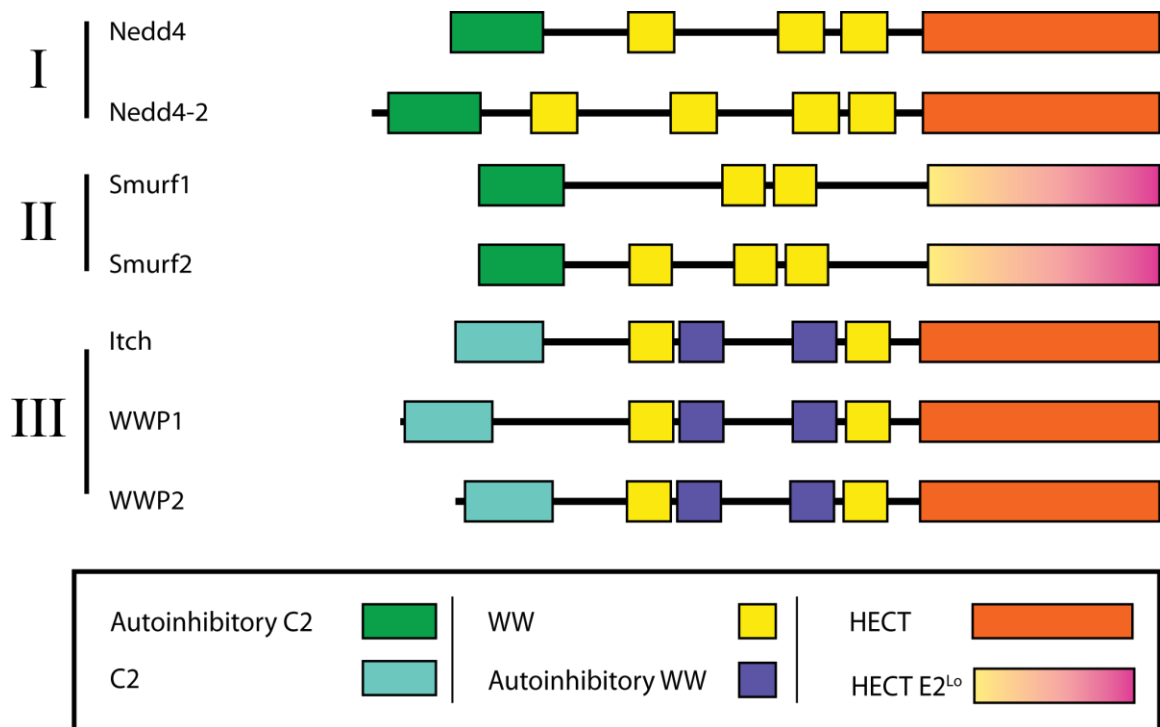


Figure 3.2: Proposed division of Nedd4 subfamilies.

Type I C2 inhibited (Nedd4 and Nedd4L), Type II E2 inhibited (Smurfs), or Type III WW domain inhibited (Itch, WWP1 and WWP2) subfamilies. While Nedd4, Nedd4L, Smurf1 and Smurf2 contain autoinhibitory C2 domains (in green), Smurfs 1 & 2 also have suboptimal E2 binding pockets in the HECT domain (HECT E2^{Lo}). Itch, WWP1 and WWP2 lack C2-mediated autoinhibition and are instead autoinhibited by their WW domains (in purple).

Nedd4 family members are involved in signaling networks with proteins containing similar domains.

How does understanding these domain contributions aid us in considering the role of the Nedd4 family in a biological context? Through a combination of their common and distinct regulatory features, the Nedd4 family is capable of both overlapping and individual action in signaling pathways. This allows the Nedd4 family to continuously interact with targets in diverse subcellular locations. Nedd4 and Nedd4L, as would be predicted, are often found to regulate events at the plasma membrane(94,96,121,188-192), where the Itch-WWP subfamily often directs ubiquitylation at the endosomal compartment(39,126,193-197). Identifying the function of Nedd4 domains, and proteins that share these domain features, provides tools to predict other components likely involved in Nedd4 family regulated signaling networks.

The C2 domain directs Nedd4 family function in membrane fusion events.

Nedd4 and Nedd4L have been extensively implicated in regulation of endocytic signaling and receptor internalization. As we would expect, considering all Nedd4 family members retain the C2 domain phospholipid interacting region, there have been multiple family members implicated in regulation of the PTEN/PI3K signaling pathway(156,198-203). Importantly, several Nedd4 family targets overlap with those of pleckstrin-homology domain containing proteins, which are also known to directly bind phospholipids. Conversely, the significance of calcium sensing among Nedd4 family members is less clear. While it has been shown that Nedd4 is indeed calcium dependent, for both localization and activation(100,119,121), the biological consequences of this dependence have not been established. However, proteins with calcium sensing C2 domains such as synaptotagmin are important regulators of vesicle fusion. The targets of these regulators of internalization events overlap extensively with those regulated by calcium-sensitive Nedd4 family members, such as internalization of EGFR or TGF β (204). It is likely that calcium binding enables Nedd4 family

members to interact with mediators of clathrin-dependent and independent calcium signaling. Accordingly, Nedd4 has been shown to bind key vesicular fusion components, mediate endocytosis, and be a critical modulator of exocytic events(89,94,121,190,205,206). In the future, studies of how calcium binding alters C2-domain mediated membrane interactions of Nedd4 family members are necessary to understand how this property contributes to Nedd4 function and why it is only seen in certain family members.

Itch-WWPs are less calcium sensitive than other Nedd4 family members.

The C2 domains of Itch, WWP1 and WWP2 also sets this subfamily apart from other Nedd4 family members. While the C2 domain of members of the Itch-WWP subfamily have been implicated in subcellular localization events, whether or not they associate with the plasma membrane has not been shown. In COS7 cells, Itch has been seen to co-localize with AP1, a marker of the *trans*-Golgi network, and mannose-6-phosphate receptor positive endosomes, known to direct membrane trafficking to the lysosomal compartment(207). In these experiments, Itch showed limited overlap with markers of the *cis*-Golgi compartment or late endosome(197). This localization was at least partially C2 dependent, as a mutant of Itch lacking the C2 domain was diffusely expressed throughout the cytoplasm. However, an experiment investigating localization of the C2 domain of Itch expressed in isolation saw no interaction with the endosomal compartment, indicating the localization of Itch is only partly determined by the C2 domain(197). I hypothesize that the Itch-WWP C2 mediates interactions with phosphoinositides at the TGN, which are known to regulate AP1 coat assembly and TGN progression(207). The WW domains are likely then required in order for Ndfip proteins to localize Itch to the endosomal compartment. These factors may allow Itch to promote lysosomal trafficking of both endosomal and Golgi-associated proteins. Additionally, I hypothesize that the Itch-WWP subfamily retains no calcium sensitivity. When compared to known calcium binding proteins such as PKC α , PLC δ 1, cPLA2 and Synaptotagmin-1, none of the Itch-WWP family members possess the highly conserved asparagine residues shown to be critical for calcium binding (Figure 3.3). In contrast, Nedd4 and Nedd4L asparagine residues align well with

the calcium binding regions of PKC α and Synaptotagmin-1, while Smurf1 and Smurf2 show alignment of only the first calcium binding region (Figure 3.3). Identification of these residues in crystal structure studies indicates that the asparagine residues of Nedd4 (Figure 3.3, in teal) overlap well with the orientation of the PKC α binding region, as does the single calcium binding region of Smurf2. Itch, however, lacks any asparagine residues at the loop region where these residues occur. Importantly, the phospholipid binding region of PKC α (Figure 3.3, in yellow) is well preserved in all Nedd4 family members. As we have seen in the case of PKC α , the ability to bind calcium can strongly alter the phospholipid interactions of a C2 domain. Thus, a characteristic that divides Nedd4 family members is likely the ability to bind, and therefore be mediated by, calcium.

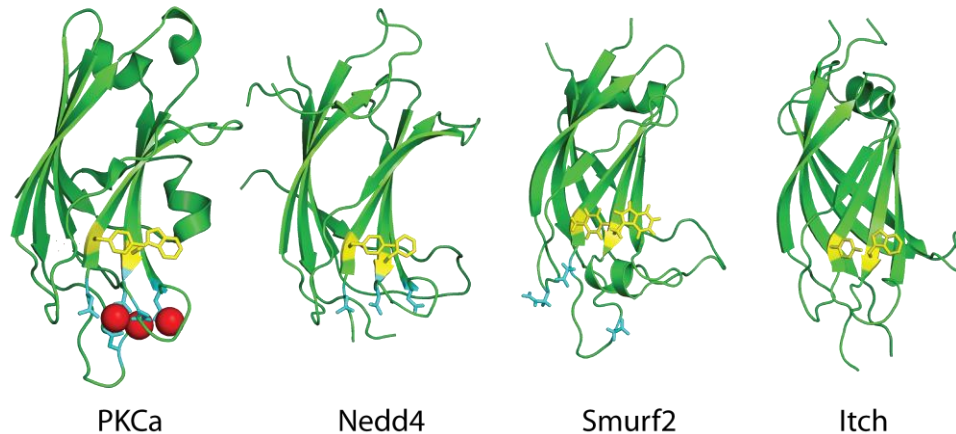


Figure 3.3: Structural analysis of the C2 domains of Nedd4 family members in comparison to PKC α . Ribbon cartoon structures of PKC α , Nedd4, Smurf2 and Itch. Red spheres represent Ca²⁺. Teal sidechains represent Ca²⁺ binding asparagine amino acids. Yellow sidechains represent phosphoinositide binding amino acids.

Nedd4 family member WW domains predict relevant signaling networks.

As mediators of substrate degradation, Nedd4 family members are often negative regulators of signaling. Since most Nedd4 family substrates are recruited by their WW domains, this negative regulation is often seen opposing positive regulators that also contain WW domains. Above, I discussed how WW domain arrangement and structure specify substrate interactions. By

identifying proteins that contain similar WW domain arrangements and structures, we can predict other proteins likely involved in Nedd4 family signaling networks.

Several Nedd4 family members have been shown to coordinate with other WW domain containing proteins. For example, in the regulation of the TGF β /Smad signaling pathway, a complex interplay between WW domain containing regulators exists. Both activating and inhibitory Smad proteins contain PPxY motifs through which they interact with WW domain containing proteins. Several Nedd4 family members act as negative regulators of Smads(128,142,159,162,208-210), while WW-domain containing proteins YAP, TAZ and WWOX are important to promoting Smad activation(141,143,211). WWOX plays a protective role, sequestering Smads from the action of Nedd4 family members in the cytoplasm and facilitating nuclear import of the Smad(143,212,213). YAP and TAZ, in the nucleus, complex with Smads in order to promote transcription(211). The balance between Nedd4s and other WW-domain containing proteins can be modulated by phosphorylating the WW domains. As described above, phosphorylation of WW domains can increase or decrease its affinity for a PY motif. This provides a mechanism by which a common domain is used to differentially regulate substrates. Another mechanism regulating the action of WW domain containing proteins is the expression of altered isoforms. WWP2 has been shown to express isoforms in which WW domains are excised, either reducing binding to Smads or resulting in a preferential binding towards a particular Smad(210). While it is not yet known whether other WW domain containing proteins use this mechanism, it is noteworthy that YAP has a naturally occurring isoform in which one of its two tandem WW domains is excised.

Signaling events involving WWOX are particularly relevant to Itch, WWP1 and WWP2. The tandem WW1-WW2 domains of WWOX align well with that of the WW3-WW4 domains of the Itch-WWP subfamily. Both WWOX and Itch have a second WW domain that possesses a tyrosine in place of the second tryptophan critical to proline-motif binding. Of the WW domain-containing proteins, this particular substitution is rare. Thus, evidence suggests that Itch will often oppose the action of WWOX on substrate. Bearing this idea out, we see several instances of WWOX and the Itch-WWP

family regulating common substrates. Both WWOX and Itch directly interact with the two p53 family members, p63 and p73(139,157,214-216). As I have discussed previously, p73 and $\Delta Np73$ are also differentially regulated by WWP2 and a WWP2-WWP1 complex(87). We also see that WWOX and Itch regulate several common substrates of Wnt and Hippo signaling, and both are critical mediators in these pathways(87,139,216-218). By identifying conserved binding regions in the WW domains and their assembly in Nedd4 family members, we can identify not only likely co-regulators and relevant pathways, but also make informed guesses regarding their physiological roles. It has already been shown that WWOX interacts with Jun family members(219). Whether we see WWOX exert a protective role in opposition to Itch in the context of JunB regulation will be an important direction for future research.

A prolyl isomerase functions as a switch for PY motif – Itch-WWP interactions.

One WW domain containing protein stands out when considering Nedd4 family member interactions with PPxY containing substrates. The WW-domain containing prolyl isomerase Pin1 is capable of interconverting *cis* and *trans* isomers of peptide bonds that contain proline(140,220,221). This can enhance or reduce the ability of a PY motif to interact with a WW domain. While the WW domain of Pin1 appears to be capable of binding both *cis* and *trans* isomers of the pT/pSPPxY motifs it interacts with equally well, the WW domains of Itch show a marked shift in affinity to these motifs once they have undergone isomerization(140,141,157). Pin1 has been shown bind and isomerize substrates in several signaling pathways that also involve Nedd4 family members, including NF- κ B signaling, p53 and p73 signaling, Notch signaling, and regulation of PTEN/Akt signaling(140,220,221). Both Pin1 and Itch recognize the p53 family member p63 through PPxY-WW domain interactions(157). The residues of WW domains 1 and 2 in Itch that contribute to this interaction align well with the phosphoserine / phosphothreonine (pS/pT) recognizing WW domain of Pin1(222). Recent work has shown that Pin1 affinity for the PPxY motif of p63 is increased when a threonine residue just N-terminal to the motif is phosphorylated. This phosphorylated form of p63 also has increased affinity for WW domains 1 and 2 in Itch. These

studies indicated that there is a critical arginine residue conserved in the WW domains of Itch and Pin1 necessary for recognizing pSPPxY/pTPPxY residues(157). We see these residues conserved in the first three WW domains of each of the Itch-WWP subfamily members (222). It is interesting to note that the third WW domain of these subfamily members, despite retaining this arginine residue, did not appear to bind to phosphorylated p63 *in vitro*. This could be an effect of the fourth WW domain acting to 'license' the third WW domain of Itch for binding much in the way that the WW domains 2 and 3 of Smurf2 interact(163). Thus, Pin1 interaction can drastically alter substrate interactions of Nedd4 family members, acting to switch PPxY motifs to preferentially interact with different WW domain containing proteins. It will also be interesting to discover whether the contribution of Pin1 is limited to substrate interactions or whether it also acts to modulate Ndfip interactions with Nedd4 family members.

The HECT domain in the future of Nedd4 family member studies.

The catalytic HECT domain is the most highly conserved domain among the Nedd4 family members. HECT domains have been seen to assemble a variety of ubiquitin linkages, including K63, K48, K33, K11, and K29 linked chains(79). How these E3 ligases generate this variety of chain linkages is unclear. While it is generally thought that the HECT domain structure determines ubiquitin chain type and length, this remains controversial. Several studies have investigated the question of K48 or K63 linked chain generation by Nedd4 family members and arrived at different conclusions, stressing the importance of further research(41,79). Despite this confusing element of Nedd4 regulation, structural studies have revealed much about how HECT domains form ubiquitin chains and what features determine their chain-building specificity. The type of chain formed by the HECT domain is greatly influenced by the C-terminal tail. A roughly 40 amino acid stretch C-terminal to the catalytic cysteine residue has been shown to strongly influence the ubiquitin linkages produced by HECT domains(79). This region is particularly well conserved among the Nedd4 family members, indicating that Nedd4-family members are predisposed to produce similar chain linkages. This C-terminal tail exhibits flex and descends into the catalytic cleft of the HECT

domain(79). To date, there are no reports of cofactors binding to this region, or post-translational modifications of this region, despite research indicating that small shifts in the C-terminal tail can result in altered chain type formation(223). The identification of potential cofactors or modifications may help us to understand the capacity for Nedd4 family members to produce such a variety of ubiquitin chains in cells, when they appear structurally constrained *in vitro*.

The significance of E2 interactions with Nedd4 family members.

The potential for contributions to Nedd4 function by E2 conjugating enzymes is still poorly understood. While transthiolation has been extensively studied in structural and biochemical contexts, it is unclear whether E2s may have additional effects on HECT type E3 action in a more cellular context. That we see Itch functioning in the presence two different E2s is worth noting. The interaction of Ubch5b/c and Ubch7 with Nedd4 family members has been described in detail, but it remains unclear whether there are functional consequences to these interactions. While the core region of these E2s is similar, their function is surprisingly different. The Ubch5 family, consisting of Ubch5a-c, displays a high degree of sequence and structural homology. Studies indicate that Ubch5 family members generally do not dictate chain topology nor appear to localize to a particular subcellular compartment(16,20,30,224). These E2s are promiscuous and not seen to discriminate substrate orientation. In contrast, Ubch7 is novel among E2s in that Ubch7 does not directly transfer ubiquitin to substrate in conjunction with RING-type E3s(225). Ubch7 is only capable of transthiolation, and so serves as an obligate partner for catalytically active E3 ubiquitin ligases(225). Interestingly, Ndfip1 specifically has been shown to recruit Ubch7 to Itch. This fits well with my data showing that Ubch7 has a much lower affinity for Itch than Ubch5b in the absence of Ndfips (Figure 2.2)(175).

Given that the purported function of E2s in interacting with Nedd4 family members is simply to provide ubiquitin, identifying whether these E2 interactions differentially affect Itch function may advance our understanding of how Nedd4 family members are capable of both mono and polyubiquitylation of substrate. Ubch5 family members have been shown to contain a conserved

non-covalent ubiquitin binding region that facilitates polyubiquitylation of substrate(224). It has been shown that other Nedd4 family members require Ubch5b and Ubch7 to ubiquitylate substrate, and that ubiquitylation mediated by Nedd4 E3s is significantly reduced in the absence of either E2(205). Interestingly, however, lacking both E2s does not decrease ubiquitylation over that of the single E2 knockdowns(205). This may suggest that in cells, it is a combination of the action of both these E2s that allows for proper substrate degradation.

Select E2s may also influence the ability of Itch to autoubiquitylate. It has been suggested that the C-terminal PY motif in the HECT domain contributes to the ability of Nedd4 family members to autoubiquitylate and subsequently signal for their own degradation. However, this action would require destabilization of the C-lobe of the HECT domain, which would disrupt catalytic activity. Therefore, it is thought that this PY motif is recognized by functioning Nedd4 family members in order to mediate degradation. Given its promiscuity, the catalytic activity of Ubch5 could provide Itch with the ability to facilitate its own ubiquitylation. In the future, selective knockdown of Ubch5 or Ubch7 in the presence of the constitutively active Itch^{WW2,3A} mutant may aid in determining the relative contributions of these E2s.

Heterodimerization and Nedd4 family members.

It is possible that Nedd4 family members bypass their restricted interactions by recruiting other E3 ligases. This may explain the conflicting results of biochemical and cell based investigations into chain specificity. The HECT domain appears to have an intrinsic RING and RING-like binding region in the N-lobe. This has been seen in the interaction of MEKK1 and Itch as well as the interaction of Nedd4 and the prototypic RING-type E3 Rbx2(226,227). Both the interaction between MEKK1 RING-like domain and the Rbx2 RING domain to the HECT domain is direct, and illustrates an interesting facet of HECT domain interactions for future study. Selective inhibition of Nedd4 catalytic activity while promoting interaction with an RING-type E3 would allow Nedd4 family members to completely alter their E2 interaction profile as well as chain specificity. This model of Nedd4 family chain specificity regulation is further complicated by the fact that Nedd4 family

members can also act in tandem with deubiquitylating enzymes to form ubiquitin editing complexes, making it difficult to discern the contributions of individual enzymes.

WWPs have also exhibited the capacity for dimerization. Regulation of p73, a p53 family member, is mediated by a novel phosphorylation induced WWP1-WWP2 interaction(87). p73 is an apoptotic regulator that is expressed in several isoforms. One of these isoforms, Δ Np73, acts in a dominant negative manner, exerting a protective effect on cell death signaling and inhibiting the action of p73's active isoforms(87). Both Itch and WWP2 negatively regulate the active isoforms of p73, apparently independently(87,217,228). WWP2 also negatively regulates Δ Np73, and more surprisingly it appears to do so in the absence of catalytic activity(87). It was discovered that WWP2 is phosphorylated by the serine/threonine kinase PPM1G, and that this phosphorylation acts as a switch, promoting the formation of a heterodimer complex with WWP2 and WWP1(87). WWP2 acts to recruit Δ Np73, and WWP1 acts to ubiquitylate Δ Np73. While it is not yet known which WW domains and phosphoresidues are involved in this interaction, the conservation of the WW domain regions seen to be involved in pS/pT binding in the Itch-WWP family indicate this form of regulation could be a facet of all Itch-WWP members. This idea would benefit from further investigation. The tools for identifying ubiquitin linkage type in a physiologically relevant context are generally thought to be less specific than required, and future studies will require a combination of biophysical and biological approaches to understand the relevance of Nedd4 family member involvement in substrate interactions.

Multiple mechanisms of activation can provide a switch to modify Nedd4 family mediated ubiquitin chain formation.

The mechanism of relief of autoinhibition of Nedd4 family members likely contributes to at least the length, if not type, of ubiquitin chain formation on their target substrate. As mentioned above, both ubiquitin chain length and type are thought to be determined by HECT domain conformations. The ability of the HECT domain to processively ubiquitylate substrate resides in a non-covalent ubiquitin-binding region within the HECT domain itself, similar to that of UbcH5(109). This binding

domain has been established both in the Smurf and Nedd4 subfamilies and has been shown to be critical to polyubiquitin chain formation(109). Importantly, residues shown to be involved in C2-mediated autoinhibition also reside on this ubiquitin binding face. In the case of Nedd4, tyrosine phosphorylation in the center of the ubiquitin binding pocket could interfere with the ability of the HECT domain to bind to the distal end of the growing ubiquitin chain, hindering its capability to polyubiquitylates substrate(229). Thus, activation of Nedd4 family members by HECT phosphorylation could promote monoubiquitylation, whereas activation through C2 calcium binding or C2 tyrosine phosphorylation would preserve the capacity of the HECT domain to polyubiquitylates substrates. How Nedd4 family members are capable of both mono and polyubiquitylation is a major question that has not, to date, been resolved. Identifying the relevance of mechanisms of activation that target the HECT are likely to be informative.

In the case of Itch, WWP1, and WWP2, identifying the region of the HECT domain involved in WW domain interactions is key. I have validated at least one of the two proposed regions that mediate autoinhibition. While structural information would indicate that this motif is buried in the native HECT conformation, the motif resides within a highly flexible hinge region. The ability of the HECT domain to interact with an E2, accept the ubiquitin protein on its catalytic cysteine, and subsequently transfer that ubiquitin protein to a substrate relies heavily on the conformational flexibility conferred by this hinge region (30,176). The N and C lobes rotate about the hinge region, moving more than 40 angstroms in order to accommodate the various positional conformations necessary for efficient transfer and ligation of ubiquitin(30,110,176,230). It is likely that the second region is a feature of the native HECT domain conformation, and will be revealed only by structural studies. It will be particularly interesting to observe whether this region also lies within the ubiquitin binding region, and whether there are previously unidentified post-translational modifications to the HECT domain that may abrogate this interaction.

Ndfips license specific Itch-WWP substrate interactions.

Nedd4-family E3 ubiquitin ligases, regardless of autoinhibitory mechanism, can be regulated by association with a small family of interacting proteins, Ndfip1 and Ndfip2. Both Ndfip1 and Ndfip2 are characterized by an N-terminal cytoplasmic domain containing three L/PPxY motifs, which are known binding motifs for WW domains. It has been shown that Ndfip1 is capable of potentiating activity of most Nedd4 family members *in vitro*, and I have found that Ndfip2 is likewise able to potentiate Itch activation in a ubiquitin charging assay. I propose that Ndfips affect Itch-WWP subfamily members uniquely relative to their interaction with other Nedd4 family members. Ndfips likely serve as pan-promoters of Nedd4 family members by recruiting them to the appropriate subcellular compartment. However, I determined found that this is not their primary function in Itch-WWP interaction. Rather, my data support that they primarily relieve autoinhibition.

The WW domains of the Itch-WWP subfamily are well conserved, both in sequence and position relative to one another. We can observe how this particular WW domain arrangement may allow for differential Itch-WWP family activity in the regulation of Smad signaling in *Drosophila*. The Itch-WWP homologue in *Drosophila*, Su(dx) (Suppressor of Deltex), illustrates us how this fourth WW domain enables Itch-WWP members to integrate two distinct signals. Like the Itch-WWP subfamily, Su(dx) contains 4 WW domains in a two-tandem-pair configuration. For all of these E3s, WW1 and WW2 have no intervening linker region, and WW3 and WW4 have a short (~8aa) linker region. They also each evolved a natural substitution in the proline-binding groove formed by the tyrosine in the second beta strand and a tryptophan in the third beta strand, in which the tryptophan residue is mutated. For Su(dx), WWP1 and WWP2 this changes the tryptophan to a phenylalanine. This fourth WW domain has been seen to be highly specific for a PY motif in Notch, and to have little interaction with generic PY motifs or that of a known Nedd4-family substrate, Smad7(134). Interestingly, WW domains 1-3 of Su(dx) are capable of interacting with both generic PY motif peptides and the PY motif of Smad7, but show little affinity for the PY motif of Notch. Despite this affinity, in its tandem configuration the binding of WW4 to the PY motif of Notch is inhibited by intramolecular interactions with the neighboring WW3(134). This inhibition is relieved when WW3 is bound to its preferred peptide, providing a novel form of signal integration in which the binding of

WW4 to Notch PY motifs will only occur if WW3 is interacting with a PY motif or other potential ligand.

When we consider that *Drosophila* Ndfip (dNdfip) has been shown to be an obligate component of Notch signaling, this provides a model for understanding adaptor mediated activation events in Nedd4 family members. *Drosophila* Ndfip contains a canonical PPxY motif, an LPxY motif and a poly-proline motif. Importantly, while dNdfip binds all *drosophila* Nedd4 family members, interaction with the Itch homologue Su(dx) was apparently preferred, followed by dNedd4 and dSmurf respectively(231). This provides a model in which tandem WW domain interactions provide an 'and' function for the programming of the enzyme, in which binding is only seen in contexts where both substrates for WW3 and WW4 are present together (Figure 3.4). Importantly, our data shows that Itch autoinhibition is not relieved by PPxY motifs in isolation, and support that Ndfip proteins not only relieve Itch-WWP autoinhibition in a manner unique among Nedd4 family interactions, but also act to license Itch-WWPs to interact with substrate. Further studies that identify the inhibitory binding region in the HECT domain of the Itch-WWP family are necessary to distinguish events regulated by activation as opposed to Ndfip induced licensing of substrate binding.

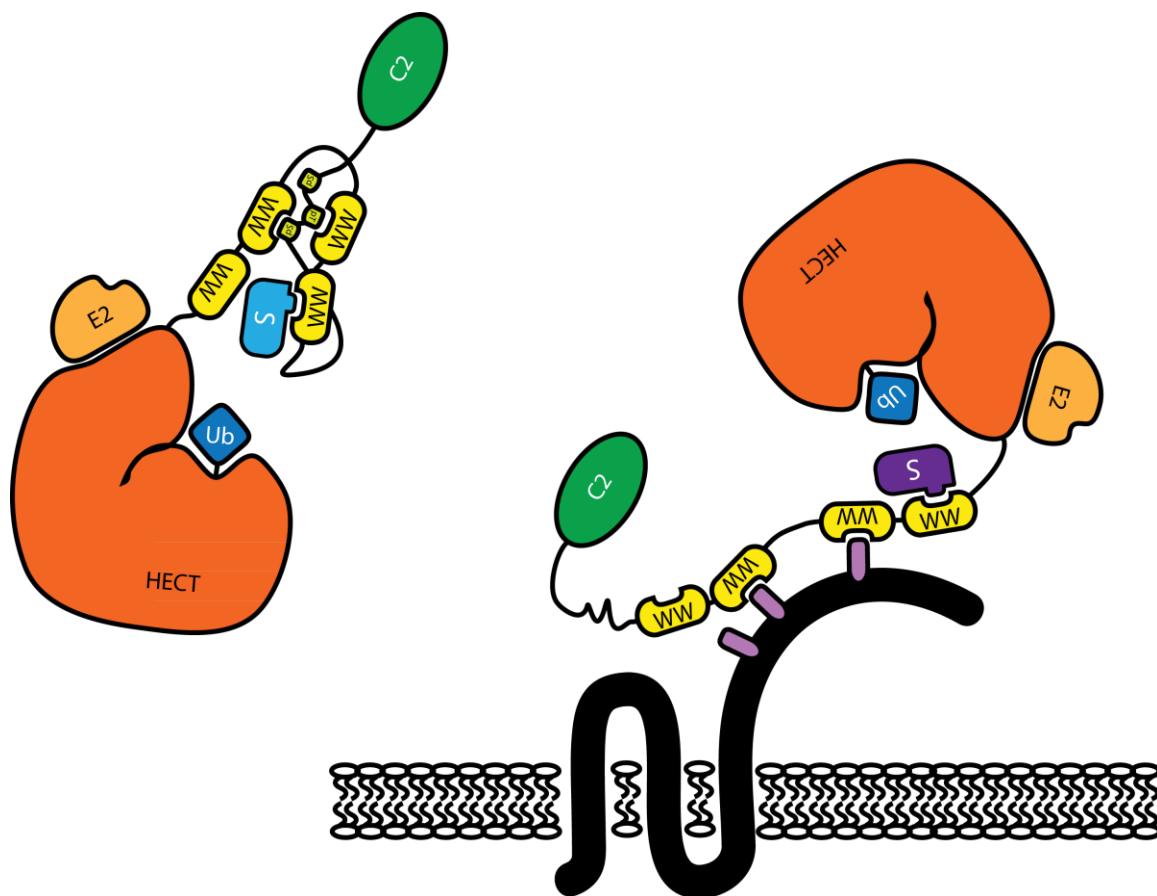


Figure 3.4: A model of Itch activation and WW domain licensing.

Left: Phosphorylation of the proline rich region of Itch promotes an intramolecular interaction in which the WW domains bind to phosphoserine / phosphothreonine residues, relieving autoinhibition. *Right:* Ndfip1 binding to the WW domains of Itch licenses WW domain 4 to bind to substrate that WW domains 1-3 are unable to interact with, ensuring that Itch only ubiquitylates certain substrates in the presence of Ndfip1.

Itch and WWP are uniquely affected by pS/pT – WW domain interactions.

Given the prevalence of pS/pT residue – WW domain interactions in the Nedd4 family, the fact that Itch is regulated by intramolecular WW domain interactions is telling. This likely makes Itch uniquely sensitive to pS/pT events. The capacity of WW domains to bind pS/pT residues integrates our data describing WW domains as mediators of inhibition and previous research investigating Itch activation. This is particularly important to consider in the context of Jnk1-mediated Itch regulation. It has been shown that phosphorylation of serine and threonine residues in a proline rich region of Itch leads to activation of Itch and subsequent degradation of the Itch substrate

JunB(104,227,232,233). The molecular mechanism of this relief of autoinhibition is unclear, however we know that Itch WW domains are able to recognize pS/pT residues, as has been seen in Itch mediated regulation of CXCR4(222). When taken in the context of my data showing that Itch WW domains mediate autoinhibition, it seems reasonable to hypothesize that Jnk-mediated phosphorylation of these serine/threonine residues results in the generation of an intramolecular binding site for the WW domains, relieving autoinhibition. In this scenario, Itch's autoinhibitory WW domains would be occupied by interactions with pS/pT residues in the proline rich region, allowing the unoccupied WW regions to interact with substrate specifically (Figure 3.4). Through such a mechanism, the cell may be able to activate Itch without recruiting it to a particular subcellular compartment (as would be the case in Ndfip interactions). Additionally, this would also provide a system by which a different array of WW domains would be available for substrate interaction, if distinct WW domains have higher affinity for pS/pT residues than PY motifs, and likewise distinct WW domains have higher affinity for PY motifs than pS/pT residues.

The regulatory role of autoubiquitylation.

My studies have also determined that Itch stability is impacted by its activation state. This would imply that Nedd4 family member autoubiquitylation serves as a failsafe mechanism that negatively self-regulates aberrantly active E3s. While all Nedd4 family members are capable of autoubiquitylation *in vitro*, the relevance of this function remains unclear. However, the markedly increased degradation of the constitutively active Itch mutant in cells, taken together with structural details on HECT-mediated ubiquitylation, provides a plausible model of Nedd4 family self-regulation. Mechanistic details from Rsp5 have shown that the distance between the PY binding motif and the target lysine of a substrate determines the efficiency of substrate ubiquitylation(176). In a PY motif interaction with WW3 of Rsp5, ubiquitylation only proceeds efficiently if the lysine residue is within a small range of amino acid residues away from the PY motif (~8-20aa). Lysine residues outside this range resulted in a shift of ubiquitylation activity by the HECT domain toward autoubiquitylation(176). As a result of this positional favoritism, substrate ubiquitylation occurs on

a timescale roughly an eighth of that of autoubiquitylation. Our data shows that constitutively active Itch autoubiquitylates itself, and that this ubiquitylation results in increased degradation. Taken together, this suggests that Nedd4 family members that are active in the absence of substrate negatively regulate themselves. This could serve both to safeguard against inappropriate ubiquitylation and as a self-regulatory mechanism that would shut down Nedd4 E3s once their targets have been destroyed.

Nedd4 family members in the balance: T cells and calcium signaling.

T cells provide an interesting confluence of Nedd4 family member regulatory events that illustrates the significance of subdividing these E3s by regulatory mechanism. Naïve T cells are activated by engagement of the T cell receptor (TCR) and the co-stimulatory CD28 receptor(146). In the absence of CD28 co-stimulation, T effector cells can become anergic(234), insensitive to stimulatory events. Upon stimulation, the T cell is required to rapidly shift from a small, relatively inactive cell to one that is rapidly proliferating and producing cytokine(145,146,235,236). This novel profile of cellular activity requires that these cells be able to survive in a relatively inert state and then dramatically shift their metabolic capacity and transcriptional programs(145). Once activated, T cells must be tightly regulated in order to ensure an appropriately directed and measured inflammatory response. The ability to swiftly terminate inappropriate activation is likewise an important feature allowing a flexible and responsive immune system. A common framework in signal transduction that promotes this balance is for regulators to mediate both positive and negative pathways downstream of stimulus(237). Ensuring a balanced response is vital to protecting this system from the deleterious effects of inappropriate anergy or chronic activation.

This can be observed in several pathways involved in T cell stimulation regulated by Nedd4 family members. For instance, calcium signaling is critical to T cell regulation, with roles in homeostasis, activation, proliferation, differentiation, and apoptosis(238). As has been described, calcium mobilization activates the Nedd4 subfamily, and Nedd4 is a known positive regulator of co-stimulatory CD28 signaling via negative regulation of Cbl-b. Calcium signaling also promotes

activation of the pro-inflammatory transcription factor NFAT(239). However, NFAT expression in the absence of AP-1 negatively regulates T cell signaling and mediates anergy and tolerance(240). Sustained calcium signaling negatively regulates T cell activation by promoting transcriptional upregulation of Itch and Cbl-b, which consequently degrade AP-1 and oppose Nedd4 signaling, respectively(241). When we consider the Nedd4 family member subsets in light of calcium sensitivity, we can hypothesize functional roles for these subfamilies. The calcium sensitive Nedd4 family may be important in immediate stimulatory signaling, when calcium mobilization is at its peak. Calcium binding to C2 domains promotes their interactions with phospholipids at the plasma membrane, and could promote not only Nedd4 activation but also localization(127). It is possible that it is important for the Itch-WWP subfamily to be either insensitive to calcium signaling or capable of action in the absence of calcium. Lastly, the Smurf subfamily, sensitive to calcium but requiring secondary effectors, may be important in events secondary to stimulation, such as differentiation or suppression. Nedd4, as has been described above, both promotes CD28 co-stimulatory signaling and negatively regulates growth factor receptors such as IGF-1R. It has been observed that activated, proliferating T cells downregulate surface expression of IGF-1R(242). IGF-1 has been shown to promote expression of the cytokine IL-10, a suppressor of inflammatory cytokine signaling, in activated T cells(243), and the IGF-1 receptor has been implicated in protecting activated T cells from apoptosis(244). Thus, Nedd4 potentially acts as a promoter of TCR signaling immediately downstream of calcium mobilization. Alternatively, Itch, as a member of the calcium insensitive subfamily, is a known negative regulator of AP-1 signaling and promoter apoptosis through regulation of c-FLIP. These calcium insensitive family members can act as attenuators of T cell responsiveness, regardless of stimulatory events. Finally, both members of the Smurf subfamily are known regulators of TGF β signaling, an important regulatory cytokine that not only suppresses inflammatory cytokine signaling but promotes T regulatory cell differentiation. T cell activation also leads to PKC θ -dependent inhibition of TGF- β signaling through Smurf1 and Smurf2(245). Interestingly, T cells do not express canonical PKC enzymes and instead express PKC θ , which possesses a calcium insensitive C2-like domain(246). In keeping with the theme of

positive and negative regulation of downstream targets through a single component, both SMURF E3s can act both as inhibitors of TGF- β signaling by inducing the degradation of Smads (247) and as positive regulators through inducing degradation of SKI-related novel protein N (SnoN), a transcriptional repressor of Smads(248). Additionally, as described previously, SMURF2 binding to the I-Smad Smad7 promotes nuclear export of the Smurf2/Smad7 complex and subsequent recruitment to the TGF- β receptor, where Smurf2 promotes the receptor's internalization and degradation(143,162). The calcium insensitive Itch family opposes this action of Smurf1/2, promoting TGF β signaling(249). Thus, the regulatory mechanisms of these Nedd4 subfamilies outline a framework that allows these highly similar proteins to distinguish their actions as temporally sensitive positive and negative regulators of T cell stimulation and differentiation.

Concluding remarks.

Data presented here, with regards to autoinhibition of Itch and its relief by Ndfip1, illustrates both a previously unknown attribute of domains found in the Nedd4 family, as well as a highly specialized regulatory element targeting these domains. The data showing that Itch autoinhibition is mediated by its WW domains allows a new model of these domains as mediators of a form of autoinhibition that is specific to a subset of Nedd4 family members, namely Itch, WWP1 and WWP2. That Itch and related family members require multiple WW domain – PY motif interactions in order to facilitate activation alters our understanding of substrate interactions, as well as when and where Itch might be relevant in cell signaling. This new information allows deeper insight into the significant elements of Nedd4 family action. By determining which regulatory mechanisms are shared among Nedd4 family members, and which are unique, we are able to predict when and where a particular type of regulatory mechanism may be relevant. Having multiple mechanisms regulating activation/inhibition and recruitment/sequestration allows for a highly adaptable and tunable response. Determining which structural elements are involved and what those elements regulate allows us to predict crosstalk with other signaling networks, where Nedd4 family action is likely to

be important, and under what physiological conditions we are likely to see Nedd4 family members as mediators of cell signaling.

Investigations into physiological Nedd4 family member action have either focused on contributions of entire domains, generally through truncation mutants, or complete proteins, through knockdown or overexpression studies. While additional *in vitro* reductionist studies that investigate isolated domains have helped illustrate their potential interactions, the ultimate function of these domains can only be understood in the context of the others present in the protein. In the future, studies investigating the physiological consequences of the attributes displayed by these domains in their larger protein context will allow a better understanding of Nedd4 family function. Structural studies of domain-domain interactions provide the information necessary to abrogate or rescue specific attributes with minimal mutations. For instance, C2 domain mutations in Itch to restore calcium binding, and in Nedd4 to abrogate calcium binding, would be key in future investigations into the significance of calcium sensitivity in Nedd4 family members. Constructs substituting the HECT domain of Nedd4 into Itch would be helpful in resolving whether WW domain autoinhibition is a property intrinsic to Itch-WWP WW domains, a combination of features unique to both Itch-WWP WW domains and HECT domains in combination, or reside specifically in the HECT domain. Additionally, the ability to target specific WW domains with small molecules may enable investigation into the concept of WW domain licensing by PY motif interactions. Importantly, the ability to make minimal mutations to Itch in order to relieve autoinhibition will allow a cleaner, more physiological investigation of the functional consequence of constitutive activation in cells.

Chapter 4 - References

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